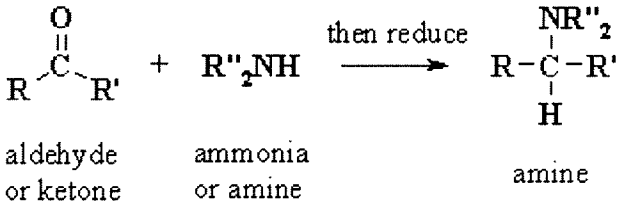
Chapter 22: Amines

Reductive Amination via Imines



Reaction type : Nucleophilic Addition then **Oxidation - Reduction**

Summary

- Aldehydes and ketones react (chapter 17) with 1° amines to give substituted imines and 2° amines to give enamines

- These N species can then be reduced to amines.
- The method provides access to 1°, 2°, or 3° amines.
- Typical reagents : **step 1** : acidic buffer **step 2** : most commonly catalytic hydrogenation (*e.g.* H_2/Pd)
- Sodium cyanoborohydride ($NaBH_3CN$) (which is like $NaBH_4$) can be used for smaller scale reductions.
- **R**, **R'** and **R"** may be either hydrogen, alkyl, or aryl.

Related reactions

- Reduction of Aldehydes and Ketones
- Reduction of Alkenes

EXHIBIT 1B

ADVANCED ORGANIC CHEMISTRY

REACTIONS, MECHANISMS, AND STRUCTURE

234567890 KPKP 783210987

This book was set in Times New Roman. The editors were Robert H. Summersgill and Anne T. Vinnicombe; the designer was Jo Jones; the production supervisor was Charles Hess. New drawings were done by J & R Services, Inc.
Kingsport Press, Inc., was printer and binder.

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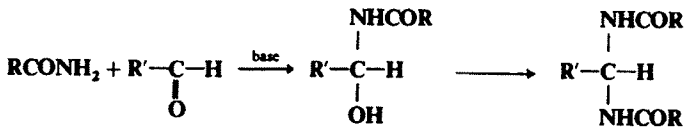
ISBN 0-07-040247-7

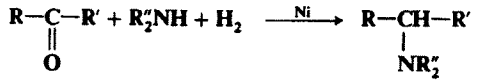
Amides can add to aldehydes in the presence of bases (so the nucleophile is actually $RCONH^-$) or acids to give acylated amino alcohols, which often react further to give alkylidene or arylidene bisamides:[111]



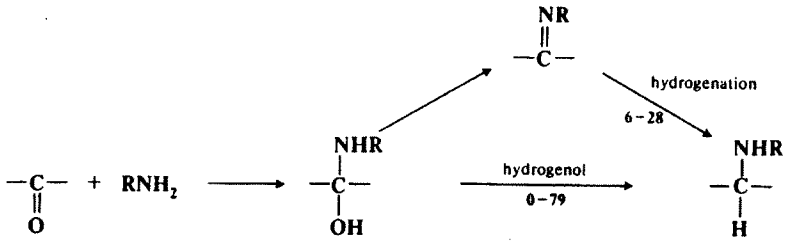
If the R′ group contains an α-hydrogen, water may split out.

OS **I**, 80, 355, 381; **II**, 31, 49, 65, 202, 231, 422; **III**, 95, 328, 329, 332, 358, 374, 513, 753, 827; **IV**, 210, 605, 638, 824; V, 191, 277, 533, 567, 627, 703, 716, 736, 758, 808, 941, 1070; **50**, 66; **53**, 44, 48, 59; **54**, 39, 46, 93. Also see OS IV, 283, 464.

6-16 Reductive Alkylation of Ammonia or Amines



When an aldehyde or a ketone is treated with ammonia or a primary or secondary amine in the presence of hydrogen and a hydrogenation catalyst (heterogeneous or homogeneous[112]), *reductive alkylation* takes place.[113] The reaction can formally be regarded as occurring in the following manner (shown for a primary amine), which probably *does* correspond to the actual sequence of steps:[114]



For ammonia and primary amines there are thus two possible pathways, but when secondary amines are involved, only the hydrogenolysis pathway is possible. Other reducing agents can be used instead of hydrogen and a catalyst, among them zinc and HCl, sodium cyanoborohydride $NaBH_3CN$,[115] sodium borohydride,[116] iron pentacarbonyl and alcoholic KOH,[117] and formic acid. When the last is used, the process is called the *Wallach reaction*. In the particular case where primary or secondary amines are reductively methylated with *formaldehyde* and formic acid,

[111] For reviews, see Challis and Challis, in Zabicky, Ref. 46, pp. 754–759; Zaugg and Martin, *Org. React.* **14**, 52–269 (1965), pp. 91–95, 104–112. For a discussion, see Gilbert, *Synthesis* 30 (1972).

[112] Markó and Bakos, *J. Organomet. Chem.* **81**, 411 (1974).

[113] For reviews, see Rylander, "Catalytic Hydrogenation over Platinum Metals," pp. 291–303, Academic Press, Inc., New York, 1967; Emerson, *Org. React.* **4**, 174–255 (1948).

[114] See, for example, Le Bris, Lefebvre, and Coussemant, *Bull. Soc. Chim. Fr.* 1366, 1374, 1584, 1594 (1964).

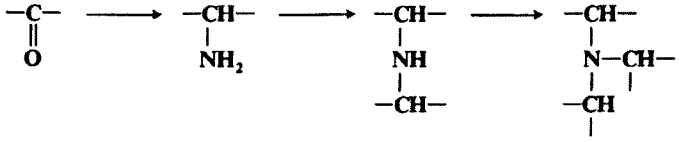
[115] Borch, Bernstein, and Durst, *J. Am. Chem. Soc.* **93**, 2897 (1971). See also Boutigue and Jacquesy, *Bull. Soc. Chim. Fr.* 750 (1973). For a review of $NaBH_3CN$, see Lane, *Synthesis* 135–146 (1975).

[116] Schellenberg, *J. Org. Chem.* **28**, 3259 (1963).

[117] Watanabe, Yamashita, Mitsudo, Tanaka, and Takegami, *Tetrahedron Lett.* 1879 (1974); Watanabe, Mitsudo, Yamashita, Shim, and Takegami, *Chem. Lett.* 1265 (1974). See also Boldrini, Panunzio, and Umani-Ronchi, *Synthesis* 733 (1974).

the method is called the *Eschweiler-Clarke procedure*. It is possible to use ammonium (or amine) salts of formic acid, or formamides, as a substitute for the Wallach conditions. This method is called the *Leuckart reaction*,[118] and in this case the products obtained are often the N-formyl derivatives of the amines instead of the free amines. Primary and secondary aromatic amines can be N-ethylated (e.g., ArNHR → ArNREt) by treatment with $NaBH_4$ in acetic acid.[119]

When the reagent is ammonia, it is possible for the initial product to react again and for this product to react again, so that secondary and tertiary amines are usually obtained as side products:



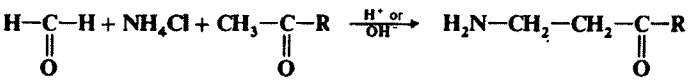
Similarly, primary amines give tertiary as well as secondary amines. In order to minimize this, the aldehyde or ketone is treated with an excess of ammonia or primary amine (unless of course the higher amine is desired).

Primary amines have been prepared from many aldehydes with at least five carbons and from many ketones by treatment with ammonia and a reducing agent. Smaller aldehydes are usually too reactive to permit isolation of the primary amine. Secondary amines have been prepared by both possible procedures: 2 moles of ammonia and 1 mole of aldehyde or ketone, and 1 mole of primary amine and 1 mole of carbonyl compound, the latter method being better for all but aromatic aldehydes. Tertiary amines can be prepared in three ways, but the method is seldom carried out with 3 moles of ammonia and 1 mole of carbonyl compound. Much more often they are prepared from primary or secondary amines.[120] The most common method for this purpose is the Eschweiler-Clarke procedure; i.e., treatment of the primary or secondary amine with formaldehyde and formic acid. Amines of the form $RNMe_2$ and R_2NMe are prepared in this manner.[121] Another method for accomplishing the conversions $RNH_2 \rightarrow RNMe_2$ and $R_2NH \rightarrow R_2NMe$ is to treat the amine with aqueous formaldehyde and $NaBH_4$[122] or $NaBH_3CN$.[123]

Reductive alkylation has also been carried out on nitro, nitroso, azo, and other compounds which are reduced in situ to primary or secondary amines.

OS **I**, 347, 528, 531; **II**, 503; **III**, 328, 501, 717, 723; **IV**, 603; **V**, 552; **52**, 124.

6-17 The Mannich Reaction



In the *Mannich reaction*, formaldehyde (or sometimes another aldehyde) is condensed with ammonia, in the form of its salt, and a compound containing an active hydrogen.[124] This can

[118] For a review, see Moore, *Org. React.* **5**, 301–330 (1949); for a discussion of the mechanism, see Lukasiewicz, *Tetrahedron* **19**, 1789 (1963).

[119] Gribble, Lord, Skotnicki, Dietz, Eaton, and Johnson, *J. Am. Chem. Soc.* **96**, 7812 (1974). See also Marchini, Liso, Reho, Liberatore, and Moracci, *J. Org. Chem.* **40**, 3453 (1975).

[120] For a review of the preparation of tertiary amines by reductive alkylation, see Spialter and Pappalardo, "The Acyclic Aliphatic Tertiary Amines," pp. 44–52, The Macmillan Company, New York, 1965.

[121] For a discussion, see Pine and Sanchez, *J. Org. Chem.* **36**, 829 (1971).

[122] Sondengam, Hentchoya Hémo, and Charles, *Tetrahedron Lett.* 261 (1973).

[123] Borch and Hassid, *J. Org. Chem.* **37**, 1673 (1972).

[124] For reviews, see Tramontini, *Synthesis* 703–775 (1973); Blicke, *Org. React.* **2**, 303–341 (1942); House, "Modern Synthetic Reactions," 2d ed, pp. 654–660, W. A. Benjamin, Inc., New York, 1972. For a review of Mannich reactions in which the active-hydrogen component is a nitro compound, see Baer and Urbas, in Feuer, "The Chemistry of the Nitro and Nitroso Groups," pp. 117–130, Interscience Publishers, New York, 1970.

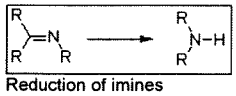
EXHIBIT 1C

Organic Chemistry Portal
Reactions > Organic Synthesis Search

Categories: C-N Bond Formation > Amines > Secondary and tertiary amines, Aryl amines >

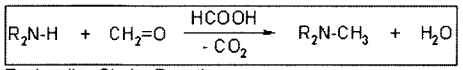
Reductive Amination

Related



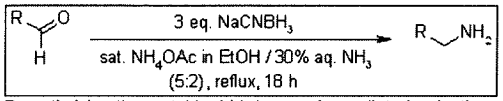
Reduction of imines

Name Reactions

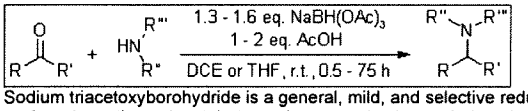


Eschweiler-Clarke Reaction

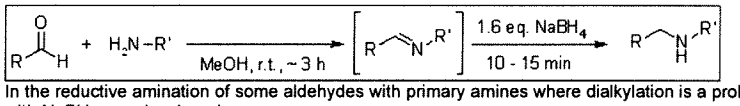
Recent Literature



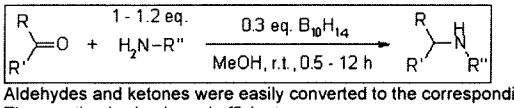
By optimizing the metal hydride/ammonia mediated reductive amination of aldehydes and hemiacetals, primary amines were selectively prepared with no or minimal formation of the usual secondary and tertiary amine byproduct. The methodology was performed on a range of functionalized aldehyde substrates, including in situ formed aldehydes from a Vasella reaction.
E. M. Dangerfield, C. H. Plunkett, A. L. Win-Mason, B. L. Stocker, M. S. M. Timmer, *J. Org. Chem.*, **2010**, *75*, 5470-5477.



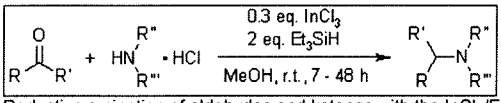
Sodium triacetoxyborohydride is a general, mild, and selective reducing agent for the reductive amination of various aldehydes and ketones. 1,2-Dichloroethane (DCE) is the preferred reaction solvent, but reactions can also be carried out in tetrahydrofuran and occasionally in acetonitrile. Acetic acid may be used as catalyst with ketone reactions. Acid sensitive functional groups such as acetals and ketals, and reducible functional groups such as C-C multiple bonds and cyano and nitro groups are tolerated.
A. F. Abdel-Magid, K. G. Carson, B. D. Harris, C. A. Maryanoff, R. D. Shah, *J. Org. Chem.*, **1996**, *61*, 3849-3862.



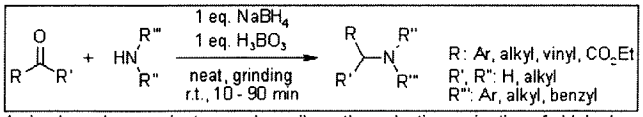
In the reductive amination of some aldehydes with primary amines where dialkylation is a problem, a stepwise procedure involving imine formation in MeOH followed by reduction with $NaBH_4$ was developed.
A. F. Abdel-Magid, K. G. Carson, B. D. Harris, C. A. Maryanoff, R. D. Shah, *J. Org. Chem.*, **1996**, *61*, 3849-3862.



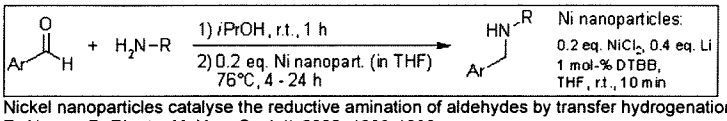
Aldehydes and ketones were easily converted to the corresponding amines by the reaction of amines in methanol using decaborane ($B_{10}H_{14}$) at room temperature under nitrogen. The reaction is simple and efficient.
J. W. Bae, S. H. Lee, Y. J. Cho, C. M. Yoon, *J. Chem. Soc., Perkin Trans. 1*, **2000**, 145-146.



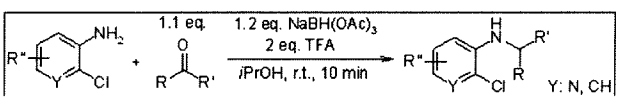
Reductive amination of aldehydes and ketones with the $InCl_3/Et_3SiH$/MeOH system is highly chemoselective and can be applied to various cyclic, acyclic, aromatic, and aliphatic amines. Functionalities including ester, hydroxyl, carboxylic acid, and olefin are tolerated.
O.-Y. Lee, K.-L. Law, C.-Y. Ho, D. Yang, *J. Org. Chem.*, **2008**, *73*, 8829-8837.



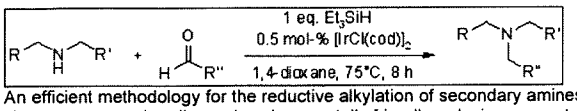
A simple and convenient procedure allows the reductive amination of aldehydes and ketones using sodium borohydride as reducing agent and boric acid, *p*-toluenesulfonic acid monohydrate or benzoic acid as activator under solvent-free conditions.
B. T. Cho, S. K. Kang, *Tetrahedron*, **2005**, *61*, 5725-5734.



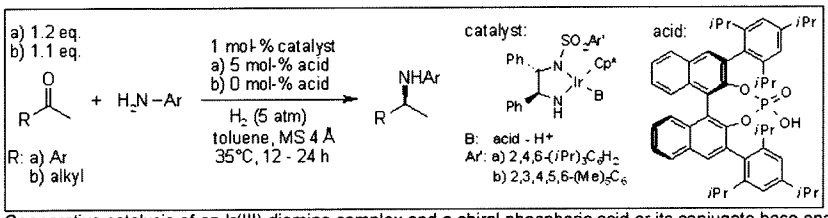
Nickel nanoparticles catalyse the reductive amination of aldehydes by transfer hydrogenation with isopropanol at 76°C.
F. Alonso, P. Riente, M. Yus, *Synlett*, **2008**, 1289-1292.



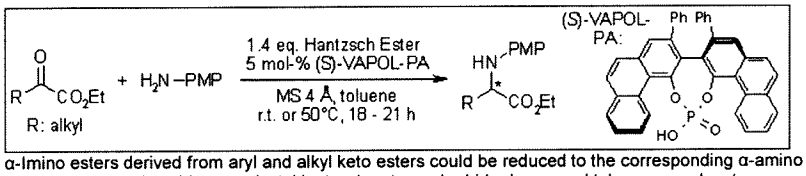
An effective reductive alkylation of electron-deficient *o*-chloroarylamines was developed. The derived *N*-alkylated *o*-chloroarylamines were elaborated to *N*-alkylazaindoles and *N*-alkylindoles via a novel one-pot process comprising copper-free Sonogashira alkynylation and a base-mediated indolization reaction.
M. McLaughlin, M. Palucki, I. W. Davies, *Org. Lett.*, **2006**, *8*, 3307-3310.



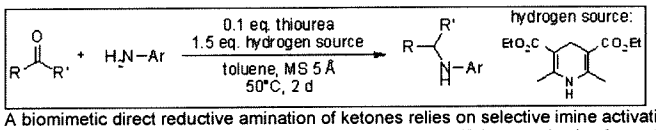
An efficient methodology for the reductive alkylation of secondary amines with aldehydes and Et_3SiH using an iridium complex as a catalyst has been developed. In addition, a cheaper, easy-to-handle, and environmentally friendly reducing reagent such as polymethylhydrosiloxane (PMHS) in place of Et_3SiH was also useful.
T. Mizuta, S. Sakaguchi, Y. Ishii, *J. Org. Chem.*, **2005**, *70*, 2195-2199.



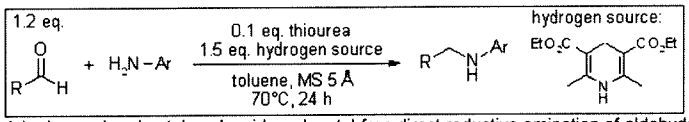
Cooperative catalysis of an Ir(III)-diamine complex and a chiral phosphoric acid or its conjugate base enables a direct reductive amination of a wide range of ketones.
C. Li, B. Villa-Marcos, J. Xiao, *J. Am. Chem. Soc.*, **2009**, *131*, 6967-6969.



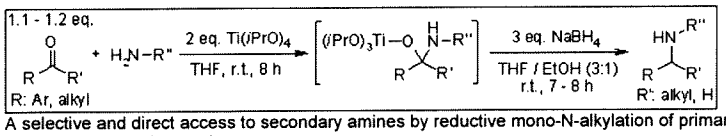
α-Imino esters derived from aryl and alkyl keto esters could be reduced to the corresponding α-amino esters in excellent yields and in high enantiomeric excesses using 5 mol-% of a chiral phosphoric acid as catalyst, Hantzsch ester as hydride donor, and toluene as solvent.
G. Li, Y. Liang, J. C. Antilla, *J. Am. Chem. Soc.*, **2007**, *129*, 5830-5831.



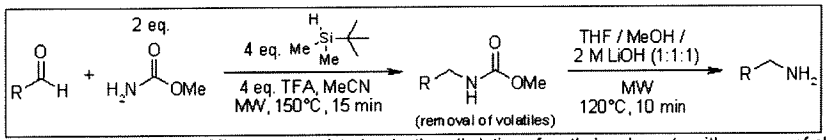
A biomimetic direct reductive amination of ketones relies on selective imine activation by hydrogen bond formation with thiourea as hydrogen bond donor and utilizes the Hantzsch ester for transfer hydrogenation. The method allows the efficient synthesis of structurally diverse amines.
D. Menche, J. Hassfeld, J. Li, G. Menche, A. Ritter, S. Rudolph, *Org. Lett.*, **2006**, *8*, 741-744.



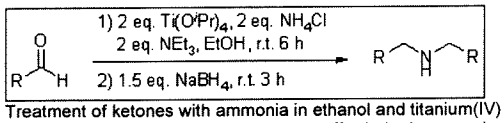
A hydrogen-bond-catalyzed, acid- and metal-free direct reductive amination of aldehydes uses thiourea as organocatalyst and the Hantzsch ester for transfer-hydrogenation. This methods allows for the high-yielding synthesis of diverse amines.
D. Menche, F. Arikan, *Synlett*, **2006**, 841-844.



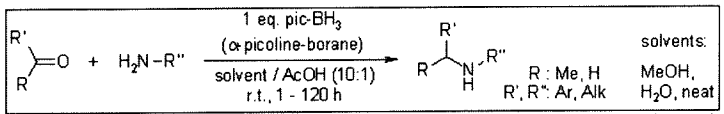
A selective and direct access to secondary amines by reductive mono-N-alkylation of primary amines with carbonyl compounds in the presence of $Ti(i\text{-}PrO)_4$ and $NaBH_4$ gave exclusively secondary amines.
H. J. Kumpaty, S. Bhattacharyya, E. W. Rehr, A. M. Gonzalez, *Synthesis*, **2003**, 2206-2210.



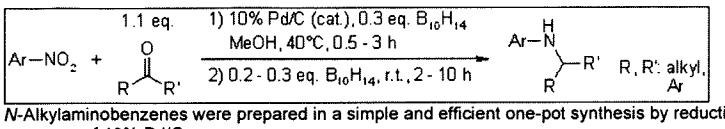
An experimentally simple Microwave-assisted reductive alkylation of methyl carbamate with a range of aldehydes provides, after basic work-up, structurally diverse primary amines. This method is particularly amenable to high-throughput synthesis.
F. Lehmann, M. Scobie, *Synthesis*, **2008**, 1679-1681.



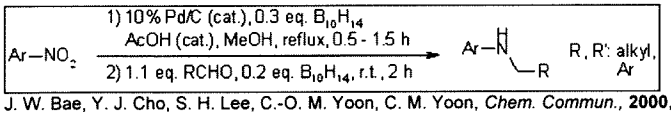
Treatment of ketones with ammonia in ethanol and titanium(IV) isopropoxide, followed by in situ reduction with sodium borohydride allows a highly chemoselective reductive mono-alkylation of ammonia. A simple workup afforded primary amines in good to excellent yields. Reductive alkylation of ammonia with aldehydes afforded the corresponding symmetrical secondary amines selectively.
B. Miriyala, S. Bhattacharyya, J. S. Williamson, *Tetrahedron*, **2004**, *60*, 1463-1471.



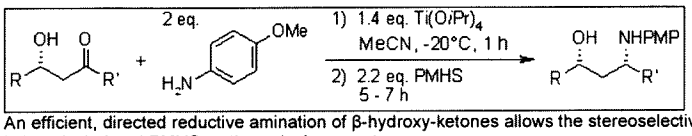
A mild and efficient one-pot reductive amination of aldehydes and ketones with amines using α-picoline-borane as a reducing agent in the presence of small amounts of AcOH is described. The reaction has been carried out in MeOH, in H_2O, and in neat conditions. This is the first successful reductive amination in water and in neat conditions.
S. Sato, T. Sakamoto, E. Miyazawa, Y. Kikugawa, *Tetrahedron*, **2004**, *60*, 7899-7906.



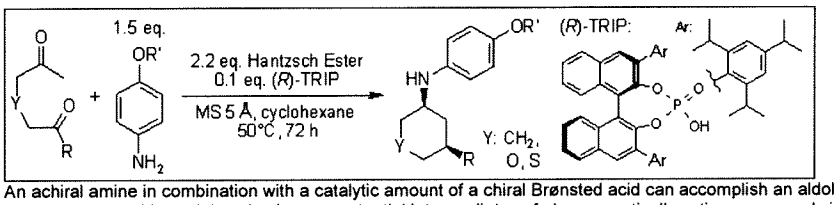
N-Alkylaminobenzenes were prepared in a simple and efficient one-pot synthesis by reduction of nitrobenzenes followed by reductive amination with decaborane ($B_{10}H_{14}$) in the presence of 10% Pd/C.
J. W. Bae, Y. J. Cho, S. H. Lee, C.-O. M. Yoon, C. M. Yoon, *Chem. Commun.*, **2000**, 1857-1858.



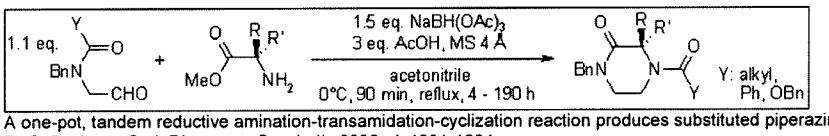
J. W. Bae, Y. J. Cho, S. H. Lee, C.-O. M. Yoon, C. M. Yoon, *Chem. Commun.*, **2000**, 1857-1858.



An efficient, directed reductive amination of β-hydroxy-ketones allows the stereoselective preparation of 1,3-*syn*-amino alcohols using Ti(*i*OPr)$_4$ for coordination of the intermediate imino alcohol and PMHS as the reducing agent.
D. Menche, F. Arikan, J. Li, S. Rudolph, *Org. Lett.*, **2007**, *9*, 267-270.



An achiral amine in combination with a catalytic amount of a chiral Brønsted acid can accomplish an aldol addition-dehydration-conjugate reduction-reductive amination with 2,6-diketones to provide cyclohexylamines as potential intermediates of pharmaceutically active compounds in good yields and excellent enantioselectivities.
J. Zhou, B. List, *J. Am. Chem. Soc.*, **2007**, *129*, 7498-7499.



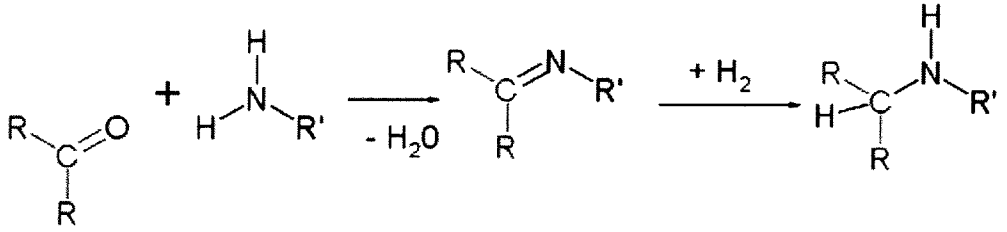
A one-pot, tandem reductive amination-transamidation-cyclization reaction produces substituted piperazin-2-ones in good yields.
D. C. Beshore, C. J. Dinsmore, *Org. Lett.*, **2002**, *4*, 1201-1204.

EXHIBIT 1D

Reductive amination

From Wikipedia, the free encyclopedia

Reductive amination (also known as reductive alkylation) is a form of amination that involves the conversion of a carbonyl group to an amine via an intermediate imine. The carbonyl group is most commonly a ketone or an aldehyde.



Contents

Reaction process

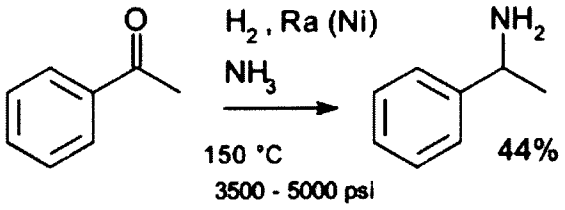
In this organic reaction, the amine first reacts with the carbonyl group to form a hemiaminal species, which subsequently loses one molecule of water in a reversible manner by alkylimino-de-oxo-bisubstitution, to form the imine. The equilibrium between aldehyde/ketone and imine can be shifted toward imine formation by removal of the formed water through physical or chemical means. This intermediate imine can then be isolated and reduced with a suitable reducing agent (e.g., sodium borohydride). This is **indirect reductive amination**.

However, it is also possible to carry out the same reaction simultaneously, with the imine formation and reduction occurring concurrently. This is known as direct reductive amination, and is carried out with reducing agents that are more reactive toward protonated imines than ketones, and that are stable under moderately acidic conditions. These include sodium cyanoborohydride ($NaBH_3CN$) and sodium triacetoxyborohydride ($NaBH(OCOCH_3)_3$).[1] This reaction has in recent years been performed in an aqueous environment casting doubt on the necessity of forming the imine.[2] This is because the loss of the water molecule is thermodynamically disfavoured by the presence of a large amount of water in its environment, as seen in the work of Turner *et al.*[3] Therefore, this suggests that in some cases the reaction proceeds via direct reduction of the hemiaminal species.[4]

Variations and related reactions

This reaction is related to the Eschweiler-Clarke reaction in which amines are methylated to tertiary amines, the Leuckart-Wallach reaction with formic acid and to other amine alkylation methods as the Mannich reaction and the Petasis reaction.

A classic named reaction is the **Mignonac Reaction** (1921) [5] involving reaction of a ketone with ammonia over a nickel catalyst for example in a synthesis of 1-phenylethylamine starting from acetophenone:[6]



In industry, tertiary amines such as triethylamine and diisopropylethylamine are formed directly from ketones with a gaseous mixture of ammonia and hydrogen and a suitable catalyst.

Biochemistry

A step in the biosynthesis of many α-amino acids is the reductive amination of an α-ketoacid, usually by a transaminase enzyme. The process is catalyzed by pyridoxamine phosphate, which is converted into pyridoxal phosphate after the reaction. The initial step entails formation of an imine, but the hydride equivalents are supplied by a reduced pyridine to give an aldimine, which hydrolyzes to the amine.[7] The sequence from keto-acid to amino acid can be summarized as follows:

$$HO_2CC(O)R \rightarrow HO_2CC(=NCH_2\text{-}X)R \rightarrow HO_2CCH(N=CH\text{-}X)R \rightarrow HO_2CCH(NH_2)R.$$

See also

- Forster-Decker method
- Leuckart reaction

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3. ^ Colin J. Dunsmore, Reuben Carr, Toni Fleming and Nicholas J. Turner, A Chemo-Enzymatic Route to Enantiomerically Pure Cyclic Tertiary Amines, J Am Chem Soc, 2224-2225, 128(7), 2006
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External links

- Current methods for reductive amination
- Industrial Reductive amination at BASF

Article published in Microscopy Today **00-1** pp. 8-12 (2000).
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Formaldehyde, formalin, paraformaldehyde and glutaraldehyde: What they are and what they do.

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The University of Western Ontario,
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Aldehydes are the most commonly used fixatives. They serve to stabilize the fine structural details of cells and tissues prior to examination by light or electron microscopy. Research workers, technicians, pathologists and others who regularly use aldehyde fixatives frequently do not appreciate the nature and properties of these compounds or the reasons for choosing to fix a specimen in formaldehyde, glutaraldehyde or a mixture of the two. Misconceptions are widespread also about formalin and paraformaldehyde, the commercial products from which formaldehyde-containing solutions are made.

Properties of formaldehyde and its polymers

Formaldehyde is a gas. Its small molecules (HCHO, of which the -CHO is the aldehyde group) dissolve rapidly in water, with which they combine chemically to form methylene hydrate, $HO\text{-}CH_2\text{-}OH$. This is the form in which formaldehyde exists in aqueous solutions; its chemical reactivity is the same as that of formaldehyde. Methylene hydrate molecules react with one another, combining to form polymers (Fig. 1). The liquid known as formalin contains 37-40% of formaldehyde and 60-63% of water (by weight), with most of the formaldehyde existing as low polymers (n = 2 to 8 in the formula given in Fig. 1). Higher polymers (n up to 100), which are insoluble, are sold as a white powder, paraformaldehyde.

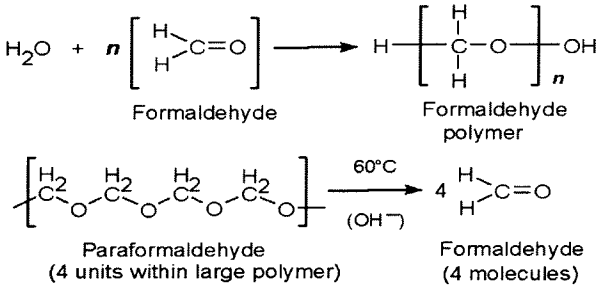

Fig. 1. Formation of formaldehyde polymers (above), and depolymerization of paraformaldehyde (below).

To be useful as a fixative, a solution must contain monomeric formaldehyde (or methylene hydrate, to be pedantic) as its major solute. Dilution with water breaks up the small polymers in formalin.

This process is said to take a couple of days if plain water is used, but to be almost instantaneous when formalin is diluted with a buffer solution at physiological pH (Pearse, 1980). Hydrolysis of the polymers is catalyzed by the hydroxide ions present in the slightly alkaline solution (Fig. 1). The big polymer molecules in paraformaldehyde need more energetic treatment. Heating is necessary, as is an added source of hydroxide ions. In one of the earliest paraformaldehyde-derived fixatives (Richardson, 1960) this was sodium sulfite, but the regular practice for at least 35 years has been simply to heat the paraformaldehyde to 60C in water containing the salts used to buffer the solution to pH 7.2 to 7.6.

Formalin contains about 10% methanol, added by the manufacturer because it slows down the polymerization that leads eventually to precipitation of paraformaldehyde. A 4% formaldehyde solution made from formalin therefore contains about 1% methanol. It also contains a small amount of formate ions. These are derived from the Cannizzaro reaction, in which two formaldehyde molecules react together, one being reduced to methanol and the other oxidized to formic acid. Because of this slow reaction, the concentrations of methanol and formate in any formaldehyde solution increase slowly with prolonged storage (Walker, 1964). A solution of formaldehyde prepared from paraformaldehyde, which does not initially contain any methanol, is commonly used in fixatives for electron microscopy and in research applications. Satisfactory ultrastructural preservation is, however, also seen in tissues fixed in buffered formaldehyde generated from formalin (Carson, *et al.*, 1973).

Reaction of formaldehyde with proteins

The aldehyde group can combine with nitrogen and some other atoms of proteins, or with two such atoms if they are very close together, forming a cross-link -CH2- called a methylene bridge. Studies of the chemistry of tanning indicate that the most frequent type of cross-link formed by formaldehyde in collagen is between the nitrogen atom at the end of the side-chain of lysine and the nitrogen atom of a peptide linkage (Fig. 2), and the number of such cross-links increases with time (Gustavson, 1956). The tanning of collagen to make leather is comparable to the hardening of a tissue by a fixative (Hopwood, 1969). The fixative action of formaldehyde is probably due entirely to its reactions with proteins. Initial binding of formaldehyde to protein is largely completed in 24 hours (Helander, 1994) but the formation of methylene bridges proceeds much more slowly. Substances such as carbohydrates, lipids and nucleic acids are trapped in a matrix of insolubilized and cross-linked protein molecules but are not chemically changed by formaldehyde unless fixation is prolonged for several weeks.

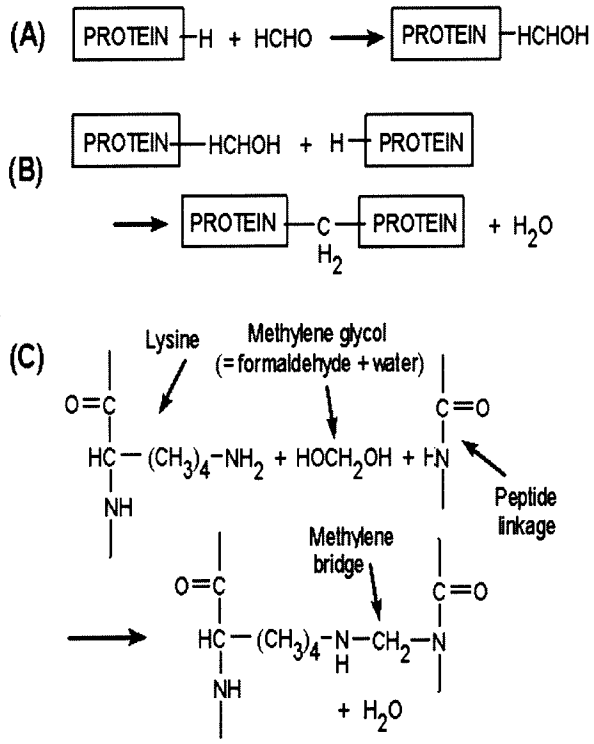

Fig. 2. Reactions involved in fixation by formaldehyde. **(A)** Addition of a formaldehyd molecule to a protein. **(B)** Reaction of bound formaldehyde with another protein molecule to form a methylene cross-link. **(C)** A more detailed depiction of the cross-linking of a lysine side-chain to a peptide nitrogen atom.

Practical considerations relating to formaldehyde

This is the most important bit. Formaldehyde penetrates tissues quickly (small molecules), but its reactions with protein, especially cross-linking, occur slowly. Adequate fixation takes days, especially if the specimen must withstand the osmotic and other stresses of dehydration and infiltration with paraffin. Brief fixation in formaldehyde (ideally delivered by perfusion) can stop or greatly reduce autolysis and confer slight hardening and some resistance (but not much) to liquids that are not iso-osmotic with the tissue. This can greatly improve the structural integrity of cryostat and other frozen sections, especially if followed by infiltration with a cryoprotectant such as sucrose (ideally 60% but more usually 15-30%).

When a specimen is dehydrated after only a few hours in formaldehyde, the largely unfixed cytoplasmic proteins are coarsely coagulated. Nuclear chromatin, which contains DNA and strongly basic proteins, is also coagulated by the solvent, forming a pattern of threads, lumps and granules. This is not unlike the appearance induced by fixatives that contain acetic acid, but it is less satisfactory for identifying cell-types on the basis of nuclear morphology. (After adequate formaldehyde fixation, chromatin displays a remarkably even texture, also of little diagnostic value but possibly closer to the structure of the living nucleus.)

Glutaraldehyde solutions

Before 1962 the only satisfactory fixative for electron microscopy was buffered osmium tetroxide. This preserves cellular structure by combining with lipids, especially in membranes, and by insolubilizing some proteins without coagulation, but it is expensive and toxic, penetrates tissues extremely slowly, and extracts much protein and RNA. With the introduction of glutaraldehyde (Sabatini *et al.*, 1962) electron microscopists had a more rapidly penetrating fixative that thoroughly insolubilized proteins and was cheap enough to deliver by vascular perfusion.

Glutaraldehyde has fairly small molecules, each with two aldehyde groups, separated by a flexible chain of 3 methylene bridges. It is $HCO\text{-}(CH_2)_3\text{-}CHO$. The potential for cross-linking is obviously much greater than with formaldehyde because it can occur through both the -CHO groups and over variable distances. In aqueous solutions, glutaraldehyde is present largely as polymers of variable size (Monsan *et al.*, 1975). There is a free aldehyde group sticking out of the side of each unit of the polymer molecule (Fig. 3), as well as one at each end. All these -CHO groups will combine with any protein nitrogens with which they come into contact, so there is enormous potential for cross-linking, and that is just what happens (Fig. 4). There are also many left-over aldehyde groups (not bound to anything) that cannot be washed out of the tissue.

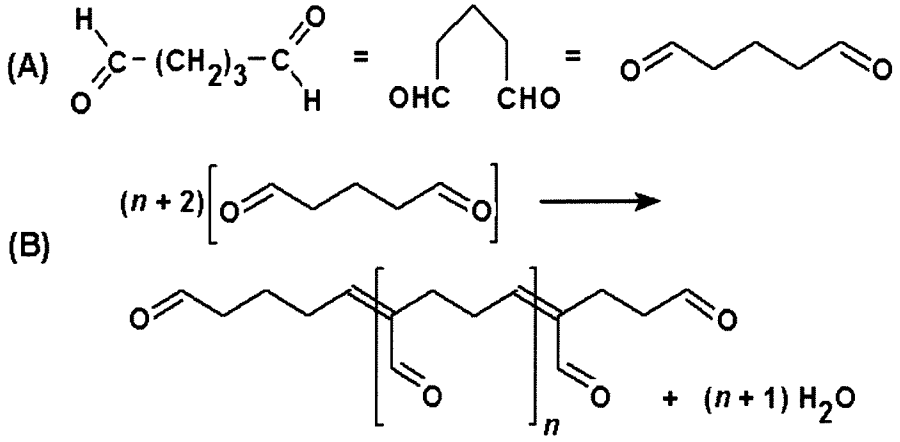

Fig. 3. (A) Three representations of a molecule of monomeric glutaraldehyde. **(B)** Polymerization reaction of glutaraldehyde, showing an aldehyde side-chain on each unit of the polymer.

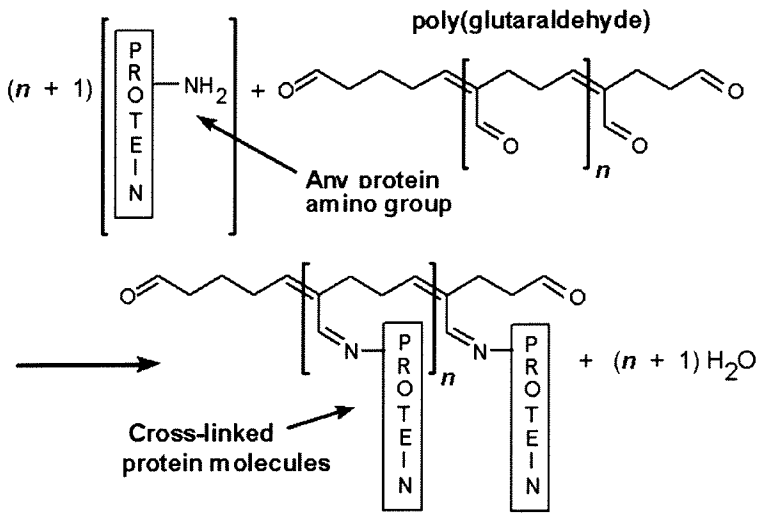

Fig. 4. Reaction of poly(glutaraldehyde) with amino groups of proteins.

Practical aspects of glutaraldehyde fixation

Five important points must be remembered when using glutaraldehyde as a fixative for light or electron microscopy.

1. If it's to be any use as a fixative, especially for electron microscopy, the glutaraldehyde solution must contain the monomer and low polymers (oligomers) with molecules small enough to penetrate the tissue fairly quickly. This means you must buy an "EM grade" glutaraldehyde (25% or 50% solution), not a cheaper "technical" grade. The cheaper stuff, which is for tanning leather, consists largely of polymer molecules too large to fit between the macromolecules of cells and other tissue components.

2. The chemical reaction of glutaraldehyde with protein is fast (minutes to hours), but the larger molecules, especially the oligomers, penetrate tissue slowly. A rat's brain left overnight in a buffered glutaraldehyde solution and sliced the next day shows a colour change and harder consistency to a depth of 2-3 mm. Objects fixed for a few hours in glutaraldehyde are no longer osmotically responsive (Paljarvi *et al.*, 1979).

3. The free aldehyde groups introduced by glutaraldehyde fixation cause various problems. These include non-specific binding of proteinaceous reagents, notably antibodies, and a direct-positive reaction with Schiff's reagent). The free aldehydes must be removed or blocked by appropriate histochemical procedures, as described in textbooks (Culling *et al.*, 1985; Kiernan, 1999, Ruzin, 1999), before attempting immunohistochemistry, lectin histochemistry, the Feulgen reaction of periodic acid-Schiff staining on glutaraldehyde-fixed material.

4. The thorough cross-linking of a glutaraldehyde-fixed specimen impedes the penetration of fairly large paraffin wax molecules. This makes for difficult cutting and peculiar differential shrinkage artifacts within the specimen. You can stain mitochondria nicely in cells surrounded by obviously abnormal spaces. This is an exaggeration of the inadequacy of formaldehyde and osmium tetroxide as fixatives to precede paraffin (Baker, 1958), and it also highlights the shortcomings of predominantly coagulant fixatives (AFA, Davidson's, Bouin etc), which preserve the micro-anatomy well but

destroy or displace little things like organelles. Fortunately, plastic monomers penetrate glutaraldehyde-fixed tissue adequately. It has been shown that they do not enter every crevice (Horobin & Tomlinson, 1976), but there is enough support to allow the cutting of ultrathin sections for electron microscopy.

5. Immunohistochemistry, which requires as many intact amino acid side-chains as possible, is severely impaired by glutaraldehyde fixation. Nevertheless, clever people have generated antibodies to individual amino acids, that are glutaraldehyde-bound to protein. These allow the detection of soluble amino acid neurotransmitters such as glutamate, GABA and even glycine in presynaptic axon terminals in glutaraldehyde-perfused central nervous tissue (Hodgson *et al.*, 1985; Hepler *et al.*, 1988; Crooks & Kolb, 1992). Extensive cross-linking also results in the loss or severe reduction of most histochemically demonstrable enzymatic activities, though several are retained after brief fixation (Sabatini *et al.*, 1962).

Mixtures containing formaldehyde and glutaraldehyde

The combination of formaldehyde with glutaraldehyde as a fixative for electron microscopy takes advantage of the rapid penetration of small HCHO molecules, which initiate the structural stabilization of the tissue. Rapid and thorough cross-linking is brought about by the more slowly penetrating glutaraldehyde oligomers. This mixture is associated with the name of Morris J. Karnovsky of Boston. It is an example of a great innovation that was published only in an unrefereed abstract (Karnovsky, 1965). His original mixture contained 4% glutaraldehyde, which was a higher concentration than many people wanted to use (Hayat, 1981). Designations like "half-strength Karnovsky" became common parlance in the 1960s and 1970s. Fixatives of this kind allowed the definitive descriptions of EM-level histology that were accomplished in the 5 or 6 years that followed the introduction of Karnovsky's fixative, and they are still routinely used.

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Number 649, 2003

EMBALMING CHEMISTRY: GLUTARALDEHYDE VERSUS FORMALDEHYDE

By: James H. Bedino, Chemist/Dir. Research
The Champion Company

Abstract: Glutaraldehyde and formaldehyde embalming chemistry and embalming action are critically evaluated and compared. The histories of both aldehydes and their uses in related fields of interest is discussed, including tanning, histology, electron microscopy, pathology and disinfection/sterilization. Similarities of reaction and result, as well as drastic differences are catalogued and delineated. An indepth discussion of the ramifications for embalmers in conjunction with a summary completes the work.

INTRODUCTION: The following article is an indepth comparison of the formaldehyde and glutaraldehyde chemical reactivities and nuances of reaction with predominately proteins and some other related chemicals and body tissues that are of importance to embalming. The comparisons are enlightening for the similarities and the vast differences between these two reactive aldehydes in protein fixation modalities. Also covered as an important topic of discussion and use is the relative disinfection and sterilization capabilities of formaldehyde and glutaraldehyde.

There has ensued for years heated argument and discussion concerning the relative abilities of formaldehyde and glutaraldehyde as embalming agents. The debate will never end, but at least the chemical facts and fictions can be brought to light. Embalmers cannot even agree on what constitutes embalming and the relative hierarchy of reasons and justifications for embalming in the first place. By this, I refer to the relentless arguments of preservation versus sanitation versus restoration, etc, etc. In the following paragraphs I will present the reported, documented and researched chemical facts and truisms regarding these aldehydes and their relative worths in embalming. It is then up to each embalmer to make a determination and choice regarding the efficacy and validity of use of the respective agents used in embalming.

A lot of the arguments in the embalming industry regarding these fixative/reactants are based on partial information, half-truths, rumors and general shock and dismay that an old friend, like formaldehyde, is questioned or called to task for any reason, valid or not. This is a fear-driven response to a situation wherein no alternatives to formaldehyde are seen, contemplated or believed to even exist. The result is a formaldehyde-apology based industry, where nothing can be questioned or indicted for any reason, for the possible result is oblivion. This fear response is most noted when formaldehyde is called into question or reported as having exposure problems and adverse health effects. The indictment against formaldehyde in regards to exposure and health is significant and to pretend otherwise is sheer folly. Glutaraldehyde has its exposure problems and health effects also, like any hazardous chemical used in embalming. The brutal truth is, that the exposure problems associated with glutaraldehyde in embalming scenarios are but a fraction of those inextricably linked to formaldehyde – and to think otherwise is foolish. The exposure and health related characteristics of the aldehydes will not be focused upon in this investigation, however. That is a topic of enormous importance and voluminous research that has and will be covered in other reports.

MANUFACTURE OF FORMALDEHYDE
Figure 1

The focus of this report is on the theoretical and practical chemistry of formaldehyde and glutaraldehyde as primarily protein precipitants or fixatives in embalming and fixation scenarios and delineation of the advantages and disadvantages accruing to each. Formaldehyde, the embalmers ancient and deadly friend, is investigated first.

FORMALDEHYDE: The credit for the discovery and first synthesis of formaldehyde generally goes to Hofmann who passed methanol/air vapors over a hot platinum wire and documented the formation of formaldehyde in 1868. There also, is a reference to Buterov in 1859 discovering formaldehyde by the attempted synthesis of methylene glycol. At any rate, formaldehyde was relatively late in isolation and synthesis compared to the analogous aldehydes (acetaldehyde, etc.) as most attempts failed due to rapid oxidation to formic acid and reaction byproducts (HCO2H→CO2 +H20). A reasonable industrial process was introduced around 1889, wherein formaldehyde in water was produced and so-named formalin became a chemical of interest.

It didn't take long for the fixation and possible disinfection properties to become evident. Most credit Blum in 1893 with the first use of 40% formaldehyde (formalin) in histology and preservation of tissues. Trillat was also, apparently involved, with remarks in 1888 and 1891 about formaldehyde action on urine causing incorruptibility and the ceasing of putridity and decay of plants and animal parts. Blum, in working with formalins in the lab, noted that his fingers hardened and dried, like alcohols would, and then experimented with this action of formaldehyde by fixing a mouse in a 10% solution and the formaldehyde embalming industry was born. The first documented embalming of a human cadaver with formaldehyde is purported to have occurred in 1899. Over 100 years later, very little is fundamentally changed in basic chemistry or technique of formaldehyde preservation of human cadavers. For the record, formaldehyde supplanted the dangerous and toxic concoctions of heavy metal salts, that were previously used with great success, by the years 1906-1910. Formaldehyde had become then, essentially, the chemical of choice for human cadaver embalming. By the late 1950's, there was purported to be over 200 varieties and variations of formaldehyde fixative solutions with all sorts of chemical additives that could be used in various branches of pathology, histology, gross anatomy, tanning of hides and embalming of specimens.

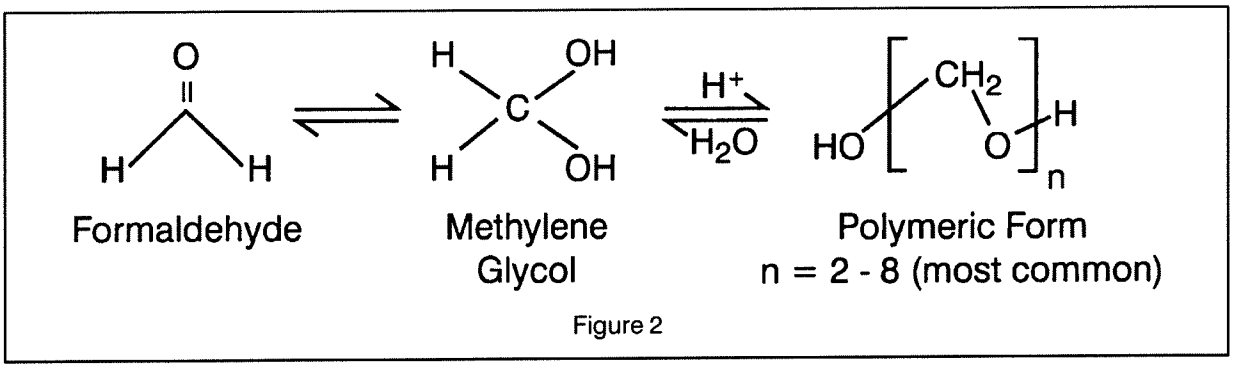
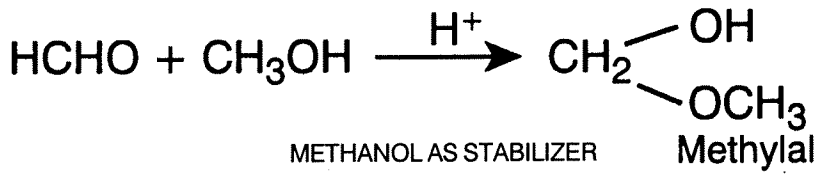


Figure 2

Modern industrial synthesis of formaldehyde is based upon oxidation of methanol over a metal catalyst (usually silver) with heat (Fig. 1). Formaldehyde is, of course, a colorless and pungently irritating gas. It is a powerful lachrimator and is explosive in air or oxygen. It is possible to liquify formaldehyde, for special circumstances of use. The vapor density is barely heavier than air at 1.06. Formalin is the chemical of commerce and is 37 to 40% formaldehyde in water by weight or volume, respectively. More dilute solutions are available and special 55% concentrations with higher alcohol content are also available. For pathology and histology uses, 10% solutions, buffered to near neutrality, are popular.

Actually, formaldehyde in formalin doesn't even exist as an aldehyde. 99.9% of formalin solutions exist as methylene glycol and its various polymers, with the true monomeric form present at only .1%.

Formalins are acidic in nature and readily form polymers of various lengths of n=2 to 100+ (Fig. 2). The smaller polymers are weak and easily hydrolyzable by acid or base hydrolysis. Polymers of n=2 to 8 are generally referred to as paraformaldehydes which exists as a white powdery solid that melts at high temperature to yield formaldehyde gas. This is a preferred way for generation of high purity formaldehyde. Aggregates of the smaller polymers can be easily hydrolyzed by water immersion and treatment by acid. The very large polymers can be difficult to hydrolyze with reaction taking weeks at a neutral pH and not much quicker even at acidic pH's. Formalin solutions are unstable and degrade if not stabilized. The most popular stabilizer is methanol at usually 3-8% in solution. Methanol rapidly forms a methylal with formaldehyde in water and is strongly favored as a reaction product (Fig. 3).



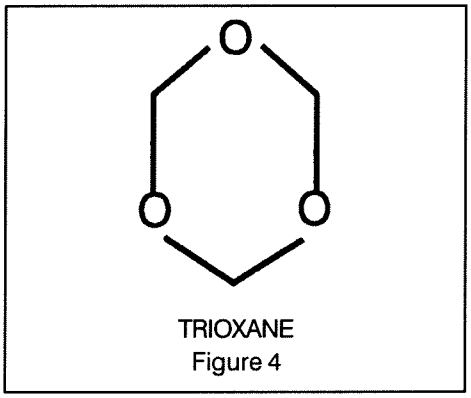
METHANOL AS STABILIZER

Figure 3

The stabilization results from an inhibition of cross-reaction and cross-addition to polymeric forms. By stabilization, up to 30% of a formalin can exist as the monohydrate methylene glycol. It is possible to utilize other stabilizers such as ethanol or even glycols or glycerine. Methanol is chosen because of its availability and it is a natural byproduct of manufacture of formalins. Other curious polymeric forms of formaldehyde can exist and be isolated, one example being trioxane, a cyclic polymer (Fig. 4). Over time, during storage, formalin solutions degrade by natural oxidation changes, caused by air or photooxidative pathways. Acidification increases, usually reaching a pH of 4 or less with the production of formic acid and formate ions. This is the reason stabilization of formalin solutions is critical. Typically, formaldehyde reactivity and polymerization increases as acidity increases, particularly at pH's of 6.5 and less. Basisity, however substantially inactivates and slows formaldehyde reactions at a pH of 8-9 and above a pH of 9 essentially non-reactive. A Cannizarro type reaction is strongly favored in very basic conditions, with a resultant loss of formaldehyde titers (Fig. 5).

Formaldehyde reaction with proteins is based on classical carbonyl-amine reaction chemistry. Amines and related nucleophiles react with formaldehyde to form various chemicals and intermediates with ultimately methylene bridging (-CH2-) resulting in fixation or tanning type action. Formaldehyde is a highly reactive carbonyl entity with no adjoining alkyl groups for stabilization, with the carbon being electrophilic and the oxygen being a nucleophilic center. Formaldehyde actually reacts as a methylene

glycol or as an unstable hemiacetal (methylal) that is strongly favored in methanolic aqueous solution. At any rate, primary amines react and form intermediate hydroxymethyl groups that drives a basisity loss with pKa drops of about 4-5 units. Subsequently, but slowly, dehydration or condensation reaction occurs by loss of a molecule of water and a methylene bridge forms (Fig. 6). Also possible, after initial reaction are dimethylene ether linkages and the reduction of hydroxymethyl groups by formaldehyde (HCHO) itself to methyl groups with production of formic acid as an endpoint product. These reducing properties of formaldehyde are accelerated in alkaline conditions where formaldehyde is known to precipitate the metals of various salts, such as bismuth, copper and silver. Basically, then endpoint reaction results are condensation with acidity promotion. Oxidation of formaldehyde can also come about spontaneously in air or with other oxidants, in addition to the hydroxymethyl groups discussed above. An interesting classical formaldehyde reaction product is that with NH3, ammonia, the reaction product being a curious cyclic, urotropin (Fig. 7).

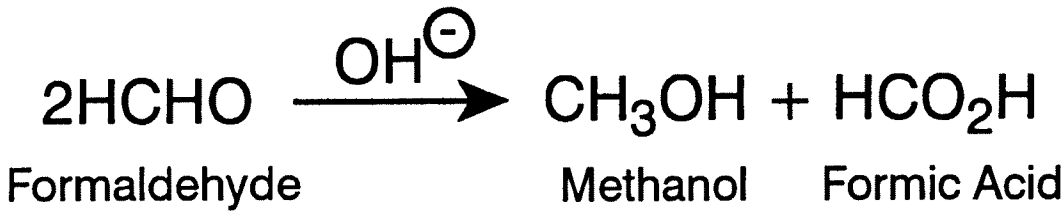


TRIOXANE
Figure 4

Specifically then, proteins present the following reactive groups to formaldehyde: terminal NH2 groupings (i.e. amines), primary amides, guanidyl groups, hydroxyls, thiols, indole nuclei, imidazolinyl and phenolic groupings. Lysine groups seem to be very preferential, probably because of conformational freedom and external surface availability as a steric effect. There are reports that thiols are not preferred and infrequently available as they are oxidized into disulfide (-S-S-) bridge linkages.

The problem with formaldehyde fixation, that has been known since 1902 is the reversibility and susceptibility to acid hydrolysis of the coagulated protein. Formaldehyde fixed gelatin was noted to be reversible by hydration and acid treatment in the very early years of formaldehyde research. In many instances, there is significant formaldehyde wash-off, i.e. unreacted or reversed formaldehyde found in post-treatment buffer wash. The amount of formaldehyde that does not wash out is reacted in a dehydration reaction. Reversing of fixation and acid hydrolysis has been know to be possible since the early 1960's by acid catalyzed hydrolysis, water immersion or heat, or a combination of the above.

The initially reversible hydroxymethyls in protein reaction, therefore, reduce by condensation reaction to hydrophobic methyls or N-formyls with formic acid formation. Methylene bridging occurs most often between lysine and various other moieties: lysine-arginine, lysine-cysteine, lysine-asparagine and lysine-glutamine and is strongly sterically controlled, occurring only when favorable proximities exist (average bonding distances being only 2 angstroms or slightly more). Dimethyl ether bridges theoretically should form but appear far fewer than anticipated.

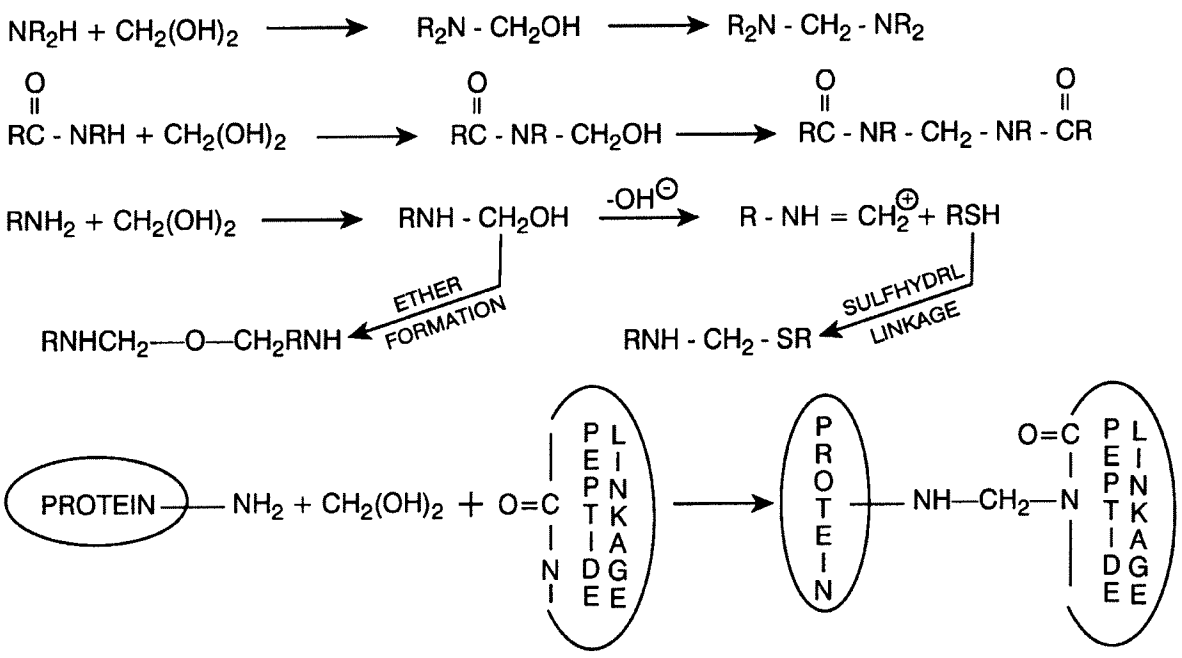


CANNIZARRO REACTION
Figure 5

In addition to the hydroxymethyl derivatives of the amine functions, guanidine, other hydroxyls, indoles and imidazoles being very unstable, certain other bridgings are also somewhat susceptible. The lysine-cysteine couplings are somewhat stable, but reversible. Lysine-arginine, lysine-asparagine and lysine-glutamine are stable but susceptible to acid hydrolysis. Lysine-tyrosine links appear to be very stable and are acid-resistant. It seems, in general that weaker and reversible links are generated during mild treatment, while strong formaldehyde treatment during fixation results in a significant amount of acid-resistant linkages. In general, pH's of 6-7 favor reversible amine reactions and pH's of 4-5.5 encourage methylene bridging. Neutral pH is not conducive to total fixation and basic pH's actually inhibit the total reaction.

Essentially, then, there are basically three competing reaction scenarios in formaldehyde fixation: 1. rapid reaction and coagulation with reversible adducts. 2. stabilized bridging but susceptible to acid hydrolysis and reversal. 3. significant endpoint bridging with high acid-hydrolysis resistance resulting in permanent fixation. The results of endpoint protein fixation are inter and intra-molecular cross-linkings causing insolubilization, trapping of various macromolecules in the fixed matrix of cross-linked proteins, dehydration and generalized hydrophobicity, and chemically induced resistance to enzyme action, microbiological interaction and chemical attack. As a side note, surprisingly and counter-intuitively, fixation does not alter the secondary structure of proteins. The more complex tertiary structures are, however, probably seriously affected by coagulation and fixation. Endpoint hardness and shrinkage of

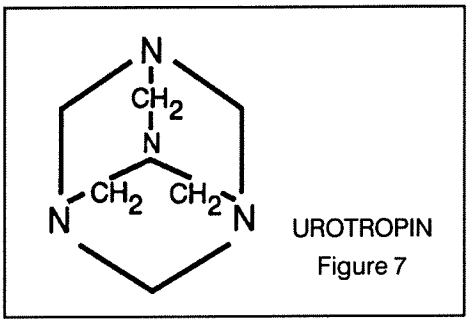
tissues is variable and slow and occurs over days. Loss of elasticity occurs and is significant but less extreme than that associated with harsher fixative methods (heat, irradiation, acids, etc.)



VARIOUS FIXATION REACTIONS OF FORMALDEHYDE
Figure 6

Reaction kinetics are therefore predicated on two distinct but separate modalities of reaction: 1. diffusion/penetration with resultant rapid but reversible product formation and, 2. endpoint fixation reaction that is extremely slow, but non-reversible and more or less a permanent fixation and precipitation of the protein. Reaction studies involving tagged C14 show actual endpoint reactions quite slow at moderate temperatures (25 degrees C) and a pH of 7. Typically 24 hours elapses before even equilibration occurs with only half or less of available reactive sites involved after 3+ hours. Reaction rate was essentially unchanged in a pH range of 3-8. Serious reaction rate reduction has been noted above this pH range. It is not unusual for essential complete dehydration reactions to require 7 days or longer and up to several weeks are not uncommon. If reaction times are only in hours, then only coarse reversible co-

agulation will occur. Formaldehyde penetration and diffusion into tissues can be tracked by the greying-out reaction of formaldehyde with colored organ tissues. For example, perfusion can be measured in liver tissue at 4-5mm in 4+ hours of immersion. In general, Fick's law is a good rule of thumb in these reactions with diffusion/perfusion proportional to the square of the elapsed time, in addition, to concentration and temperature considerations. Another curious color reaction of formaldehyde is with blood perfused tissues. At pH's of 6 or less, formal pigments of a brownish nature appears which probably is a hematin acid (probably linked to a reduction and deironization of heme). It has been found that neutral buffered or basic formalin does not cause this color reaction.



UROTROPIN
Figure 7

Little, if any reaction occurs with lipids, except possibly the amine group carrying phospholiplids. Non-saturated fatty acids are theoretically capable of reaction. Nucleic acids are reactive with various exocyclic bases such as adenine, guanine and cytoseine and endocyclic imines to form various adducts.

Occasionally additives are used to enhance tissue fixation and can include phenol at 2%, which accelerates fixation, reduces shrinkage and inhibits formal pigment production (essentially acting as a bleaching agent). Various salts of heavy and transition metals are very reactive and precipitate protein. ZnSO4, zinc sulfate, has been used with formaldehyde in immunochemistry preservation. Both tannic acid and mercaptoethanol have been utilized in electron microscopy as enhancers and enablers in conjunction with formaldehyde.

GLUTARALDEHYDE: The first successful synthesis of glutaraldehyde is credited to Harries and Tank in 1908. Glutaraldehyde was cataloged as a typically reactive dialdehyde and was used for various chemical syntheses of more complex chemicals in laboratories and its properties were moderately investigated. It was relatively difficult to synthesize in substantial quantities and was more a chemical of laboratory and synthetic chemistry interest. By the 1940's and 50's, it became obvious that glutaraldehyde exhibited properties that were superior in many ways to formaldehyde in protein fixation chemistry and the early field of disinfection/sterilization.

A successful method of industrial production was patented in the late 1950's by a Diels-Alder type reaction of acrolein and vinylethyl ether forming 2-ethoxy-3, 4-dihydro-2H-pyran which readily hydrolyzes to glutaraldehyde with a by product of ethanol (Fig.8). Interest in glutaraldehyde peaked in the early 1960's when several investigations found it to have outstanding disinfection and sterilization capabilities, even surpassing formaldehyde, the standard of the disinfection industry at the time. By 1963, high-level disinfectants, cold-chemical sterilants and potent sporicides were marketed with glutaraldehyde as the active ingredient. Interest has been intense throughout the years for glutaraldehyde, right up to the present, as it still is essentially the gold-standard for chemical forms of sterilization. All disinfectants and new alternative disinfectant chemicals efficacy are based upon comparison to glutaraldehyde efficacy.

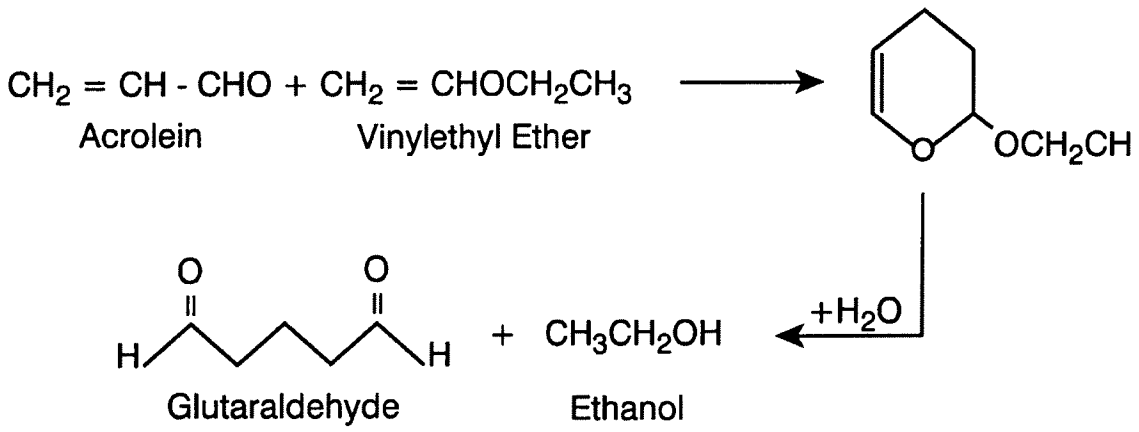


Figure 8

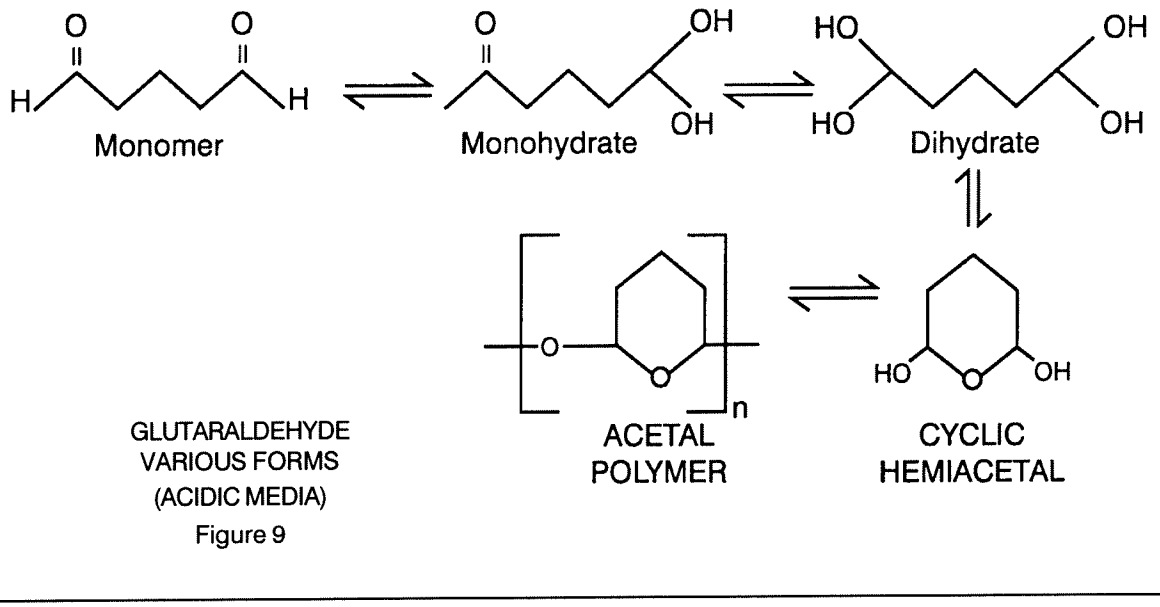
Glutaraldehyde rapidly was accepted and preferred in electron microscopy and pathology/histology labs as a superior alternative to formaldehyde. Tissue sections showed less distortion, brittleness, shrinkage, more total fixation on concentration/time frames and maintained elasticity during manipulation and sectioning, in addition to having longer shelf life. Glutaraldehyde was introduced into the embalming industry, essentially, by the Champion Company in the early 1960's through several patented formulations of glutaraldehyde and glutaraldehyde/formaldehyde based arterial, cavity and accessory chemicals.

Glutaraldehyde has also achieved high levels of acceptance and preferred use in the leather and hide tanning industry. Glutaraldehyde is the preferred aldehyde-based tanning chemical in the United States, virtually replacing formaldehyde, the older chemical standard. Glutaraldehyde tanned hides and leath-

ers exhibit better elasticities, less evidence of brittleness and cracking with wear, more suppleness and extended life, all of which creates higher levels of consumer acceptance.

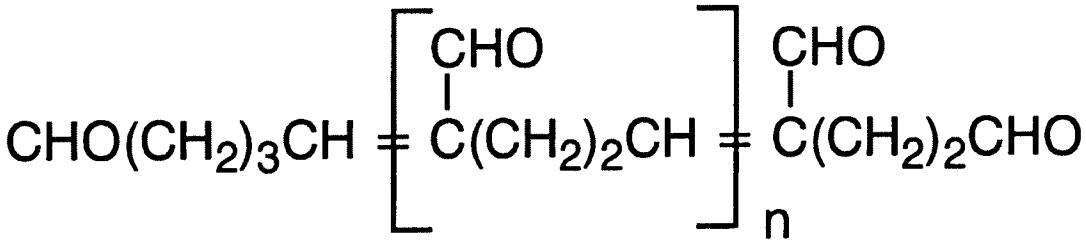
Commercially, glutaraldehyde is typically available in 2%, 25% and 50% solutions in water, with other dilutions occasionally seen and used. In acidic media, which is typically how glutaraldehyde is supplied to users, glutaraldehyde, being a highly reactive aldehyde, exists as a mixture of hydrated and non-hydrated forms. Therefore, monomer, open-chain mono-hydrates and di-hydrates, a cyclic hemiacetal and an acetal polymeric form all exist in a complex equilibrium. In acidic conditions, the cyclic hemiacetal and the acetal polymers of varying chain length are the preferred form and predominant (Fig. 9).

In neutral to basic media, this is not the case as glutaraldehyde spontaneously undergoes a self aldol-condensation reaction and dehydration to form α, β-unsaturated aldehyde polymers of varying chain length (Fig. 10). Effects of pH on reactivity demonstrate a steady increase of activity from pH4 to pH9, with maximum reactivity around pH of 8 or so. Above a pH9 there is a general decline in reactivity to pH11, after which little reaction capability is noticed. Precipitation type polymers are not common but can occur. Heated solutions demonstrate trimers, pentamers and heptamers with a trioxane skeleton and paraglut has been characterized as being 2, 4, 6-tris (4-oxobutyl)-1, 3, 5-trioxane, the polymeric form responsible for the white precipitate sometimes seen in stored glutaraldehyde solutions (Fig. 11).



GLUTARALDEHYDE
VARIOUS FORMS
(ACIDIC MEDIA)
Figure 9

In reaction with proteins the aldol polymers of glutaraldehyde react to form α, β-unsaturated imino type reaction products that are highly resonance-stabilized and very resistant to acid hydrolysis and rehydration (Fig. 12). Schiff base type reaction products that would be susceptible to acid hydrolysis and rehydration apparently do not form or survive during reaction. Epsilon-amino groups, (e.g. lysines) are particularly reactive and sterically accessible to glutaraldehyde. In addition, lysine residue analysis show pKa values of 8-8.5 (noted as a slight acid shift in the alkaline titration range) which seems to confirm the formation of michael-type adducts of aldol polymers that are acid-hydrolysis resistant. Schiff base type adducts would show pKa's of less than 5 to 6 and demonstrate susceptibility to rehydration, which is not the case with glutaraldehyde protein fixation. Another proposed bridged reaction product is a 1, 3, 4, 5-substituted pyridinium salt, similar to desmosine, an amino acid (Fig. 13).

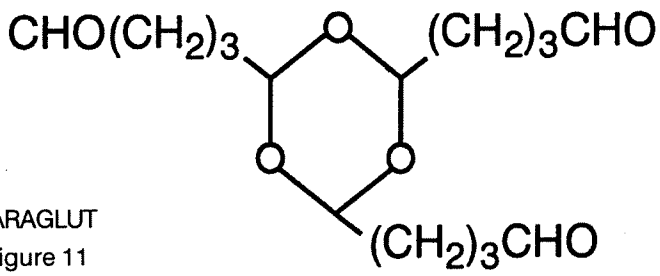


GLUTARALDEHYDE
ALDOL - TYPE POLYMER
Figure 10

From studies in the tanning industry, where acidic glutaraldehyde is the standard, effective fixation and cross-linking obviously occurs without a preponderance of unsaturated aldol-type reactants, indicating that bridging and cross-linking effectively occurs with unsaturation in acidic conditions.

In reaction, glutaraldehyde forms amino-methylols and then furthur condenses with other groups, such as phenolics, imidazoles, indoles, sulfhydryl of cysteine and to form bridged linkages. Very reactive sites appear to be terminal amine groups, α-amino groups of amino acid peptides, cysteine (via the -SH terminus), while imidazoles appear reactive but less preferred. Studies verifying high reactivity show 90% of free amino groups were reacted in 2 hours at pH6-7 with glutaraldehyde, while only 70% were reacted in formaldehyde at 7 days at a temperature of 35 degrees C. Cross-linking of proteins also occurs to a significant degree with studies of ovalbumin/bovine serum albumin aggregation showing 88% effectiveness in cross-linking.

In electron microsopy, OsO4 (osmium tetroxide) was the preferred fixation agent prior to the advent of glutaraldehyde. Glutaraldehyde was rapidly accepted as a superior fixative in this specialized field. Studies confirmed extremely fast reaction rates for glutaraldehyde (measured in minutes and hours) in contrast to formaldehyde which required days for complete endpoint fixation. Residual active - CHO (aldehyde) groups were found to be numerous after fixation and must be neutralized before processing into sections. This abundance of residual aldehyde moietites explains the time delayed fixation effects noticeable in glutaraldehyde treated tissues. Penetration and diffusion rates for glutaraldehyde are very slow with typical values of 2-3+mm of penetration on rat brain overnite. This is in sharp contrast to formaldehyde, which exhibits fast penetration/diffusion rates but very slow endpoint fixation rates. In fact, glutaraldehyde/formaldehyde mixtures are being employed as an effective fixative that combines the best attributes of both aldehydes. Glutaraldehyde reaction with lipids and nucleic acids is as expected based on aldehyde chemistry and is similar to that of formaldehyde.

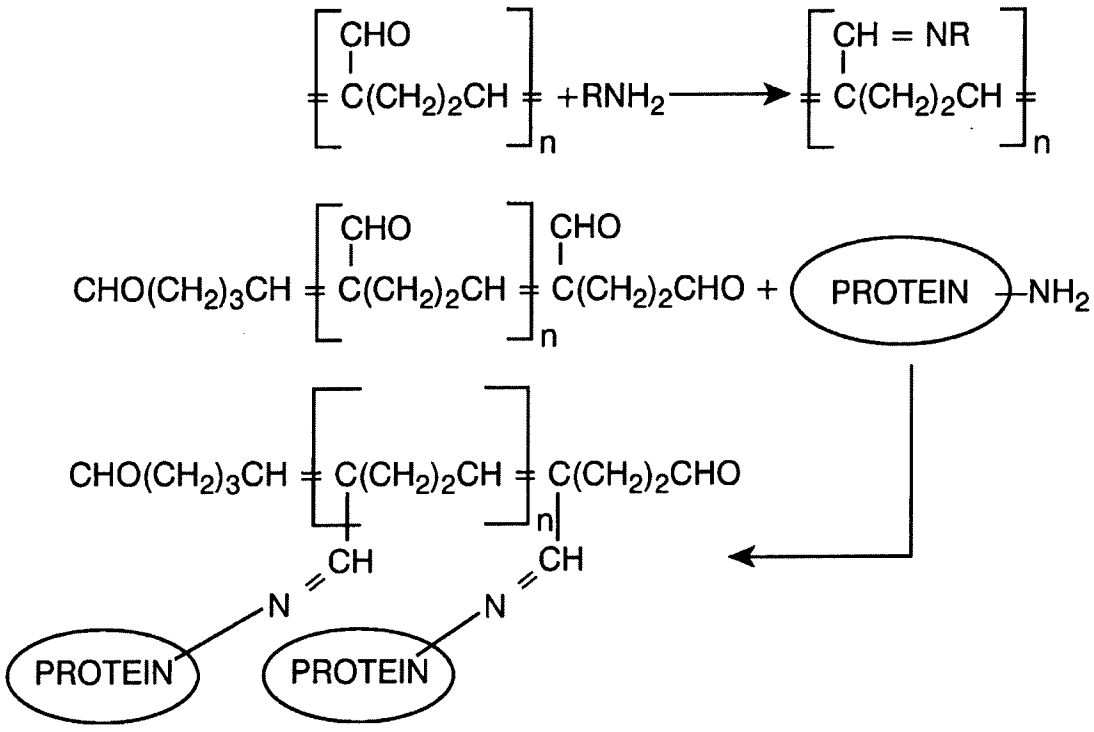


PARAGLUT
Figure 11

DISINFECTION/STERILIZATION: Formaldehyde is classified as a good high-level disinfectant and under most circumstances of use sporicidal as well. Effective concentrations range from a minimum accepted of 5% to typically 8% for consistent high-level disinfection/sterilization with time frames of action from a minimum of 3 hours to typically 8+ hours. Formaldehyde has been used for years in these disinfection scenarios with good results. Formaldehyde is a proven consistent and effective disinfectant in almost all clinical and industrial settings.

With the introduction of glutaraldehyde to the medical field, in the early 1960's, formaldehydes' popularity and usage has declined to very low levels (barely 5% of the total medical aldehyde disinfection/sterilant market). Almost no one markets a formaldehyde medical sterilant anymore in the U.S. Formaldehyde usage is more common in Europe, but still at fractional levels of what glutaraldehyde and alternatives are used at. Gigasept is a formaldehyde containing disinfectant that is used in Europe, but it has a helper aldehyde added for efficacy. Also popular in Europe, but seriously waning, is LTSF (low temperature steam formaldehyde) as disinfection/sterilization for heat-sensitive materials. Other

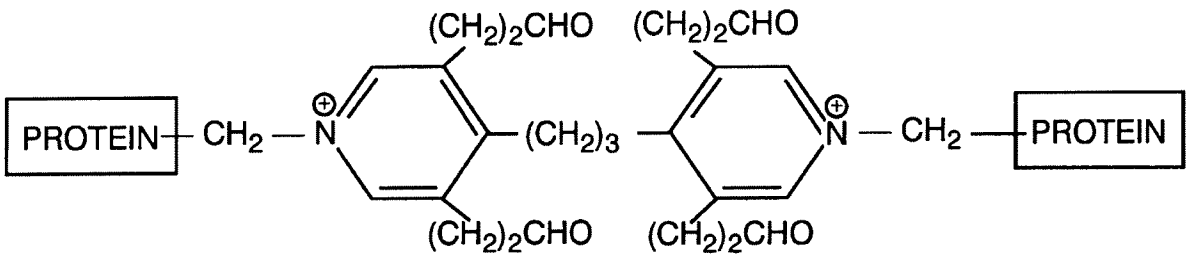
uses, still popular, are as a gas fumigant for biological safety cabinets and rooms, a gas bomb fumigant agent as a pesticide or disinfectant (generated usually by heating paraformaldehyde), and in poultry farms for incubators. Formaldehyde is also used as a general and effective, low-cost disinfectant and sanitizer for chicken coups, hog barns and dairy farms.



GLUTARALDEHYDE CROSS - LINKING PROTEIN REACTIONS
Figure 12

Glutaraldehyde is classified as an excellent high-level disinfectant/cold-chemical sterilant under almost all scenarios in which it is employed. Glutaraldehyde has, essentially, replaced formaldehyde as the premier aldehyde based disinfectant/sterilant. Glutaraldehyde is considered the gold standard to

which all other similar disinfectants/sterilants are compared. Glutaraldehyde consistently demonstrates efficacy at 2% concentrations for high-level disinfection and sterilization. Glutaraldehyde is superior to formaldehyde in both concentration and time factors—essentially demonstrating 2-4 times effectiveness at drastically reduced time exposures. Glutaraldehyde at 2% easily equilibrates to classical formaldehyde solutions of 5-8% and with equal or reduced contact times and capability of organic debris loading. Some modern glutaraldehydes are available at higher concentrations (2.8 and 3.2%) for faster cycle times, higher debris load, longer solution life and certainty of efficacy. Heated solutions are now popular to reduce contact times and insure sterilization.



GLUTARALDEHYDE LINKED REACTANT
Figure 13

EMBALMING COMPARISONS: From the above discussion, it is obvious that glutaraldehyde and formaldehyde are reactive aldehydes with significant protein fixation capability and embalming action. Formaldehyde is a fast diffuser and gives a rapid but reversible reaction with proteins. Glutaraldehyde, on the other hand, is a slow diffuser but delivers a rapid and non-reversible final reaction with proteins. Therefore, glutaraldehyde is expected to deliver more endpoint permanent fixation but perfuse the tissues slowly, while formaldehyde perfuses tissues rapidly but only forms irreversible fixation at a very slow rate. These facts result in very definite advantages and disadvantages of the respective aldehydes as embalming agents of human cadavers.

There are definite advantages in the use of formaldehyde for embalming. A rapid perfusion occurs which results in a fairly quick and noticeable coarse protein coagulation with early tissue firmness, which is easily observed by the embalmer. In conjunction with this rapid coagulation is a considerable dehydration reaction, mostly reversible but slowly converting to irreversibility over time. Dehydration is also encouraged by the natural wash-out of unreacted formaldehyde from tissues, which could be described as a type of secondary dilution. Formaldehyde, therefore is a very effective embalming agent

where fast reaction and dehydration ability is important, as in the case of edematous bodies.

Formaldehyde, unfortunately, has many drawbacks which weigh against its notable fast initial reactivity and ability to overcome edema. Actually, the rapid diffusion of formaldehyde and early reaction, that so impresses embalmers, is the cause of serious embalming problems. Too fast of a reaction results in the formaldehyde walling-off effect, formaldehyde burn, the wash-out effect and tissue shrinkage in non-edematous bodies. The result of high reactivity and rapid diffusion, therefore, hinders rathers than helps the overall embalming with sometimes disasterous effects. Bodies become very effectively superficially or shell-embalmed, but perfusion to underlying and deeper tissues is impeded or eliminated, resulting in a serious embalming problem 48-72 hours later. Everybody has embalmed a body with high index fluid to rock hardness that later, inexplicably, softens and shows classic signs of decomposition. What's the explanation for this?—rapid superficial embalming with little or no deep tissue perfusion and fixation which reverses and proceeds to a decomposition state.

Another major drawback is formaldehyde's inability to effectively embalm in a highly alkaline pH range. Many bodies currently encountered in embalming are saturated with nitrogenous byproducts of various disease states (e.g.-renal/hepatic failure, jaundice, pancreatic cancers, cancers in general, chemotherapy bodies, high titers of antibiotics and high blood levels of numerous therapeutics) in addition to being feeble and debilitated. Formaldehyde is at a serious disadvantage, in these situations, and sometimes extraordinary quantities of formaldehyde embalming agents are necessary to overcome this neutralization and inactivation effect.

The rapid reaction of formaldehyde also results in problems with clearing of blood-engorged tissues. The blood gravelling effect of formaldehyde is significant, and unless controlled and buffered, will result in poor clearing of superficial tissues during embalming with resultant staining evident. Ashen-greying is another serious unavoidable problem with formaldehyde embalming. Unless concealed by dyes or cosmetics, formaldehyde embalmed tissues exhibit a very unappealing death pallor, best described as putty-grey. This coloration is notorious in conjunction with formaldehyde embalming with poorly formulated, high index fluids with ineffective buffering and control agents.

Glutaraldehyde has many advantages to offer in embalming, the most important being relative imperviousness of reaction rate to pH changes, particularly in the alkaline range. Glutaraldehyde will react with protein at higher pHs that would essentially render formaldehyde inactive. Because of slow diffusion/perfusion rates, reaction with blood and blood-perfused tissues is slow with minimum initial coagulation. Clearing of blood from tissues is, therefore, strongly enhanced relative to formaldehyde.

There is minimal or no walling-off effect, tissue burning reaction or superficial rapid embalming as would be typical with formaldehyde. Cosmetic effects of embalming is noticeably better than with formaldehyde, with no greying or ashen-whitening effect noticeable. Tissues overembalmed with glutaraldehyde will show a yellowish-tanning coloration and severe overembalming will significantly darken tissues. Fortunately, these effects are easily overcome, in most instances, with tissue dyes during injection. A very important advantage of glutaraldehyde over formaldehyde is in regards to sanita-

tion potential. Ounce for ounce, glutaraldehyde, through its superior disinfection/sterilization capabilities, which are well documented in the medical field, is far more effective as a sanitizing agent in embalming than formaldehyde. Formaldehyde can only equal this sanitizing ability by the use of an overwhelming concentration, which is counterproductive to good embalming results.

The major disadvantage of glutaraldehyde in embalming is slow diffusion/perfusion rate. This results in most of the classical signs of embalming appearing very late or not at all to the embalmer during the embalming. The bodies will not stiffen and harden rapidly, if at all, dehydration and skin tightness will not be evident, and the bodies will usually display a lifelike appearance that, to the embalmer, belies the confirmation of embalming. In addition, the embalmed body will further firm and harden over time and possibly darken as delayed additional fixation occurs. This physical symptomology is opposite that noted in formaldehyde embalming, against all precepts taught in embalming training, and counter-intuitive to the average embalmer. Glutaraldehyde infused bodies appear nothing like traditional formaldehyde embalmed corpses. Invariably, rigidity is minimal, skin elasticity is extreme, dehydration is non-existent, lifelike appearance is typical, and flexibility is considerable. Most embalmers would interpret this set of observables as evidence of lack of embalming. Actually, it is no more than evidence of lack of sequelae of formaldehyde embalming and nothing more.

This leads us, inevitably, to the precepts of the formaldehyde-apology industry. Basically, the arguments are this: 1. there is nothing besides formaldehyde on the face of the earth that is usable by the funeral industry for the embalming of dead human bodies, 2. therefore, formaldehyde is not to be questioned or called to task for any reason (health effects, exposure dangers, embalming efficacy or otherwise), 3. Under all circumstances, formaldehyde is to be justified as safe and effective in the embalming industry, for to do otherwise is to stare into the abyss. The inevitable fallout of this paradigm is the accusation that glutaraldehyde does not embalm or fix tissues.

This is a difficult one to profess. Chemical aldehyde reactivity goes hand-in-hand with sanitizing/disinfecting/sterilizing ability, tanning/fixation ability and histology/pathology fixation. To profess that, yes, glutaraldehyde is a proven tanning chemical, yes, glutaraldehyde is a proven aldehyde disinfectant/sterilant, yes, glutaraldehyde is a proven histology/pathology fixative, but, alas, glutaraldehyde does not embalm tissue -- is patently absurd. This tack is understandable but untenable.

One example may serve to enlighten. I personally have embalmed approximately 1000 bodies with a certain chemical (JaunDial) which contains a mix of glutaraldehyde and formaldehyde with the usual buffers, penetrants and control chemicals characteristically found in modern embalming fluids. The percentage of formaldehyde present in JaunDial is barely 4.0%, which means in my 1000 embalmings of typically normal and jaundice bodies (some of which were held up to 2 weeks before interment), the amount of formaldehyde in the total embalming was approximately 3/4 ounce. Therefore, I am led to believe that the typical 175 pound human cadaver, which demonstrated excellent preservation and cosmetic effect, in almost all cases, was solely due to the influence of 3/4 ounce of formaldehyde?!

Anatomical cadavers and specimens have been infused with glutaraldehyde embalming fluids and remain preserved after years, viscera has been treated with Cavity 48, a glutaraldehyde-based cavity fluid with no formaldehyde, the body then buried and disinterred after a year with evidence of extreme mummification and petrification present. Countless glutaraldehyde infused and saturated specimens exist in labs across the globe after decades. Hundreds of tons of leather products exist that were fixed/tanned by glutaraldehyde—how is it possible that these preserved animal skins are candidates for decomposition after decades of use and wear? The documentary and evidentary list goes on and on. I will not belabor the point, but to say, that the accusation that glutaraldehyde does not embalm is untenable.

What is possible is a poorly embalmed body resulting from a poorly planned and executed embalming using a poorly formulated glutaraldehyde injection chemical—so what else is new?—this happens all the time in traditional formaldehyde embalming. Where do the decomposed bodies in disinterments, putrefaction in delayed burials, persistent skinslip, tissue gas cases, and all the miasmatic mausoleums come from? Virtually all can be traced to a traditional formaldehyde embalming. All preservatives/fixatives, aldehydes or otherwise, will fail under certain circumstances of use, due to a constellation of chemical inhibition, physical parameters of infusion, unwanted cross-reactions and bad luck. No embalming chemical is immune from these causes of poor embalming results.

SUMMARY AND CONCLUSION: Formaldehyde and glutaraldehyde each have much to offer to the modern embalming industry. Formaldehyde excels in certain embalming scenarios, such as edema, and presents rapid and classical results familiar to embalmers. Glutaraldehyde excels at sanitizing ability, mildness of reaction, tissue clearing, cosmetic effect and prolonged, delayed action. Glutaraldehyde is excellent in cavity fluids where contact times are long and resistant fixation is essential. In arterial injections of typically normal bodies, pre-injection of glutaraldehyde followed by formaldehyde injection to rapidly induce hardness of tissue, or injection of glutaraldehyde/low formaldehyde mixes, to moderate reaction, yield excellent results. It is shocking how little formaldehyde is needed to yield classical signs of embalming (firmness, rigidity, skin tightness) in normal body injections (typically 4-6 ounces in the last part of the injection of a moderate concentration [index 20] fluid). Injections that stress and take advantage of the inherent advantages of both aldehydes yield the consistently best embalming results. The overall goal of chemical selection mix in the modern embalming room should be a juxtaposition of lowered total overall exposure risk balanced against sufficient and effective embalming results, but that is a topic for another day.

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EXHIBIT 3A

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Regulation of Cell-Substrate Adhesion by Proteoglycans Immobilized on Extracellular Substrates*

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We have demonstrated previously that chick embryo fibroblasts synthesize and secrete a large chondroitin sulfate proteoglycan (designated PG-M) that binds to fibronectin. We now report the possibility that PG-M interactions with cell surfaces can modulate cell-substrate adhesion. When PG-M was added to the medium, various types of trypsinized cells failed to adhere not only to fibronectin-coated substrates but also to collagen- or vitronectin-coated substrates. Adhesion of the cells to laminin or glycyl-arginyl-glycyl-aspartyl-serine derivatized serum albumin (arginyl-glycyl-aspartic acid-containing molecules with no capacity to bind PG-M) was also inhibited by PG-M. Treatment of the proteoglycan with either proteolytic enzymes or chondroitinase abolished its inhibitory effects on the cell adhesion. These results suggest that direct binding between PG-M and fibronectin, if any, is not a cause of the inhibition by PG-M and that only the proteoglycan form is responsible for the activity. When the immobilization of added PG-M to available plastic surfaces of coated dishes was blocked by pretreating the dishes with serum albumin, the inhibitory effect of PG-M was abolished, suggesting that the immobilized fraction of PG-M can act as a cell adhesion inhibitor. In immobilized form, both cartilage chondroitin sulfate proteoglycan (designated PG-H) and chondroitin sulfate-derivatized serum albumin also inhibited cell adhesion. In contrast, heparan sulfate proteoglycan form LD and heparan sulfate-derivatized serum albumin had far lower inhibitory activities, indicating that the active site for the interaction between cells and PG-M is on the chondroitin sulfate chains.

Adhesion of cells to extracellular matrices is a fundamental process in the formation and maintenance of animal tissues and body morphology. Molecular events involved in such cell-substrate adhesion have been to some extent elucidated by recent studies on cell-substrate adhesion molecules and their cell surface receptors (for reviews, see Refs. 1–8). For example, fibronectin has been shown to interact with integrins, integral plasma membrane protein adhesion receptors, many of which can recognize the specific peptide sequence RGD[1] in cell attachment domains of fibronectin, vitronectin, and several other proteins (7, 8). Certain synthetic peptides containing this sequence have also been shown to interact with such receptors, producing either competitive inhibition or induction of cell-substrate adhesion. Additionally, another critical structure besides RGD exists in fibronectin that is essential for full adhesive function (9). In contrast, YIGSR, a peptide sequence in the B1 chain of laminin, has been shown to be a site recognized by a laminin receptor that is distinct from the RGD-recognizing receptors (10). The receptors appear to associate with some cytoskeletal components (11, 12) and plasma membrane components (13, 14), which together constitute the molecular machinery mediating cell-substrate adhesion.

During cell division, translocation, and migration, there is likely to be inhibitory regulation of cell-substrate adhesion. As possible mechanisms for such regulatory steps, proteolytic or glycolytic degradation of extracellular adhesion macromolecules (15, 16), phosphorylation of integrin (17), and proteolysis of talin by a calcium-dependent protease (18) have been postulated. Since extracellular matrices are composed of various types of collagens, proteoglycans, glycosaminoglycans, and glycoproteins (for reviews, see Refs. 19–21), it appears likely that some of them participate in the regulation of cell-substrate adhesion. Some glycoproteins have indeed been shown to inhibit cell adhesion onto substrates (22, 23). In addition, some proteoglycans have been implicated as regulating factors in cell-substrate adhesion (24–30). Here we analyze more directly the regulatory function and site of action of a large chondroitin sulfate proteoglycan from chick embryo fibroblasts, PG-M (31), in modulating the adhesion of cells to various adhesive proteins.

EXPERIMENTAL PROCEDURES

Materials—All chemicals of reagent grade were purchased from Nakarai Chemicals, Kyoto, Japan; DEAE-Sephacel and Sephadex G-50 were from Pharmacia Japan, Tokyo; Hanks' balanced salt solution, Dulbecco's phosphate-buffered saline, Dulbecco modified Eagle's medium, and Eagle's minimal essential medium were from Nissui Sei-

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[1] The abbreviations used are: RGD, arginl-glycyl-aspartic acid; PG-M, type M proteoglycan (fibroblast type, see Refs. 31 and 52); PG-H, type H proteoglycan (cartilage type, see Ref. 37); GRGDS, glycyl-arginyl-glycyl-aspartyl-serine; YIGSR, tyrosyl-isoleucyl-glycyl-seryl-arginine; REDV, arginyl-glutamyl-aspartyl-valine; CEF, chick embryonic fibroblast; BHK, baby hamster kidney; CHO, Chinese hamster ovary; EHS tumor, Engelbreth-Holm-Swarm tumor; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

yaku Co., Tokyo. Trypsin (2 × crystalized) was from Boehringer Mannheim-Yamanouchi, Tokyo. Soybean trypsin inhibitor (type 1-s), bovine serum albumin (A-4378), heparin (type I from procine intestinal mucosa), and phenylmethanesulfonyl fluoride were from Sigma. Tissue culture dishes were from Beckton Dickinson, Oxnard, CA; 96-well microtitration polystyrene plate was from Sumitomo Chemical Co., Tokyo; fetal calf serum was from Commonwealth Serum Laboratories, Melbourne, Australia; and bovine serum was from Nakarai Chemicals. Chondroitin sulfates derived from whale and shark cartilage, chondroitin (a chemically desulfated product of whale chondroitin sulfate), heparan sulfate from porcine lung, hyaluronic acid from cockscomb (average molecular weight 43,000), chondroitinase ACII, and chondroitinase ABC (protease-free grade) were the products of Seikagaku Kogyo Co., Tokyo, and were kindly donated by Mr. M. Mizutani of the company. Pronase was a gift from Kaken Chemicals, Tokyo. Peroxidase-conjugated anti-mouse IgM (μ-chain-specific) goat IgG was purchased from Tago Inc., Burlingame, CA; peroxidase-conjugated anti-rabbit IgG (heavy and light chain-specific) was from Cappel, West Chester, PA. Monoclonal antibody MO-225 (32) and rabbit anti-rat plasma fibronectin antiserum (33) were prepared and characterized as described previously.

Tertiary cultures of chick embryonic fibroblasts (CEF) were established as described previously (34) and maintained in Eagle's minimal essential medium supplemented with 10% (v/v) fetal calf serum. BHK-21 cells (baby hamster kidney cells) and CHO-K1 cells (chinese hamster kidney cells) were kindly provided by Dr. R. Ishida and Dr. C. Sato, respectively, Aichi Cancer Research Center, Nagoya, Japan. B16F10 high metastatic melanoma cells generated by Dr. I. J. Fidler were a gift of Dr. E. A. Davidson, The Pennsylvania State University, Hershey, PA. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum.

Type I collagen was isolated from rat tail tendon as described previously (35). Mouse and human plasma fibronectin were purified using gelatin and heparin affinity columns (34). Human vitronectin prepared by the method of Hayman *et al.* (36) was donated by Mr. T. Uejima in this laboratory. PG-H, the major proteoglycan of chick embryo cartilage, was purified from the epiphysial cartilage of 17-day chick embryos (37). Heparan sulfate proteoglycan form LD, the low density form of heparan sulfate proteoglycan (38), laminin, and type IV collagen prepared from the EHS tumor were donated by Dr. M. Kato in this laboratory. GRGDS pentapeptide was purchased from Peptide Institute, Inc., Osaka, Japan. Fragments of human fibronectin were prepared as described previously (39).

PG-M, the large chondroitin sulfate proteoglycan produced by chicken fibroblasts, was isolated by a modification of the previously described method (31). Briefly, chick embryonic fibroblasts were cultured to confluency in a roller bottle (850 cm^2 surface area/bottle). Two days after confluency, the conditioned media were collected continuously for up to the 3rd week when the cells began to display lipid droplets and were discarded. All operations described below were performed at 4 °C. To the collected medium, phenylmethanesulfonyl fluoride, EDTA, and Tris-HCl, pH 7.4, were added to 2, 5, and 20 mM, respectively, and the solutions were centrifuged at 25,000 × *g* for 15 min to remove cell debris. Solid NaCl was then added to the supernatant solutions to make 0.25 M, and a 500-ml portion of each solution was applied to a DEAE-Sephacel column (40 ml) equilibated with 0.25 M NaCl, 1 mM phenylmethanesulfonyl fluoride, 5 mM EDTA, 20 mM Tris-HCl, pH 7.4. The column was washed with 200 ml of the buffer and then eluted with 100 ml of 6 M guanidinium chloride, 1 mM phenylmethanesulfonyl fluoride, 5 mM EDTA, 20 mM Tris-HCl, pH 7.4. When the culture was metabolically labeled with [^{35}S]sulfate, about 80% of the labeled macromolecules in the medium was recovered in the eluate. The eluate was concentrated to 20 ml by ultrafiltration on an Amicon P-10 membrane and then mixed with solid CsCl to give a final density of 1.47 g/ml. Dissociative CsCl isopycnic centrifugation at 105,400 × *g* was carried out in a Hitachi RP-55T rotor at 10 °C for 50 h, and the bottom fractions with a density ≧ 1.50 g/ml were pooled and dialyzed successively against 1 M NaCl and distilled water. Protein content was determined by the methods of Lowry *et al.* (40) using bovine serum albumin as a standard and hexuronate content by the method of Bitter and Muir (41) using glucuronolactone as a standard. In a typical experiment, a PG-M preparation containing about 1 mg of hexuronate and 0.3 mg of protein was obtained from 100 ml of initial culture medium. Treatment of the proteoglycan fraction with chondroitinase ABC (protease-free, a special preparation donated by Seikagaku Kogyo, Co.) followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a doublet of the core molecule bands (M_r 550,000 and 500,000) (31) plus two bands (M_r 120,000 and 90,000) corresponding to the enzyme used. The PG-M sample thus obtained was capable of binding to hyaluronic acid, fibronectin, and type I collagen as ascertained by the methods described previously (31).

Cell Adhesion Assay—Cell-substrate adhesion was assayed by the method of Ruoslahti *et al.* (42). Briefly, cells cultured in 100-mm dishes were rinsed with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline, and treated with 5 ml of 0.1 mg/ml trypsin in the same buffer at 37 °C for 5 min (for BHK, CHO, and B16F10 melanoma cells) or for 15 min (for CEF). Trypsin was inactivated by the addition of 5 ml of 1 mg/ml soybean trypsin inhibitor in the above buffer and then removed by centrifugation. The cells were further washed with trypsin inhibitor-containing solution twice. The single cells thus obtained were suspended in Hanks' balanced salt solution supplemented with 20 mM HEPES, pH 7.4, and number of cells was determined with a hemocytometer. Ninety six-well polystyrene plates were coated with various proteins in 0.1 M $NaHCO_3$ (100 μl/well) and incubated at 20 °C for 2 h. Details of the method for protein coating are described in each figure legend. The protein-coated plates were rinsed with Dulbecco's phosphate-buffered saline three times, and each well was then filled with 100 μl of Hanks' balanced salt solution supplemented with 20 mM HEPES, pH 7.4, containing test substances. An aliquot (1×10^4 cells/100 μl) of the single cell suspension was added to each well, and the plates were incubated for 2 h at 37 °C. After unattached cells were removed by two washes with Hanks' balanced salt solution, attached cells were fixed in 2% (w/v) formaldehyde in phosphate-buffered saline at 4 °C for 10 min and then stained with 1% (w/v) toluidine blue/3% (w/v) formaldehyde in phosphate-buffered saline. The number of cells in five different randomly chosen areas was determined at 200 × magnification with an Olympus transmitted light microscope for the attachment index. The assay conditions employed here were designed to find usually 80-95% of seeded cells attached to the plates which had been coated with 5 μg/ml fibronectin.

In the experiments where the effects of PG-M or related substances on cell-substrate adhesion were examined (see "Results"), polystyrene plates coated with fibronectin (or related substances) as described above had residual uncoated plastic adsorption sites. These available spaces were subsequently coated with either bovine serum albumin (control) or a test proteoglycan as follows. To a plate that had been coated with limited amounts of fibronectin (usually 5 μg/ml of fibronectin) was added 200 μl (per well) of 1% (w/v) bovine serum albumin in phosphate-buffered saline that had previously been heated at 90 °C for 5 min. The plate was incubated at 20 °C for 30 min and then rinsed with Hanks' balanced salt solution. The fibronectin on the plate was quantitated by enzyme-linked immunosorbent assay (39) using rabbit anti-rat plasma fibronectin antiserum (1:100 dilution) with peroxidase-conjugated anti-rabbit IgG goat second antibody (1:1000 dilution). PG-M bound to the plate was quantitated by the enzyme-linked immunosorbent assay with MO-225, described previously (31).

Binding of Chondroitin Sulfate Proteoglycan to Fibronectin and Fibronectin Fragments—The original method described previously (30) was modified as follows. For the protein coating of plates, 50 mM Tris-HCl, pH 7.4, was used in place of the carbonate buffer. For the detection of substrate-bound chondroitin sulfate proteoglycans by enzyme-linked immunosorbent assay (43), the monoclonal antibody MO-225 against chondroitin sulfate chains (30) was employed. The antibody concentrations used were 1:1000 dilution of MO-225 ascites fluid and 1:500 dilution of the second antibodies, peroxidase-conjugated goat anti-rabbit IgG.

Enzymatic Treatment of PG-M—PG-M (100 μg as hexuronate) was incubated with either 10 μg of protease (L-1-tosyl-amido-2-phenylethyl chloromethyl ketone-treated trypsin or Pronase) or 0.1 unit of chondroitinase ACII at 37 °C for 1 h in 1 ml of Hanks' balanced salt solution. The protease treatments resulted in complete disappearance of the proteoglycan band according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Coomassie Blue staining. Treatment with chondroitinase ACII, on the other hand, resulted in appearance of core protein bands of M_r 550,000 and 500,000. The reactions for proteolytic enzymes were terminated by incubating at 100 °C for 5 min. These conditions for enzyme denaturation did not alter the activity of untreated intact PG-M molecules in inhibition of cell-substrate attachment (see "Results").

Preparation of GRGDS-derivatized Serum Albumin—The fibronectin cell-binding sequence GRGDS was cross-linked to bovine serum albumin (molar ration of peptide to serum albumin = 50:1) by a modification of the glutaraldehyde-promoted Schiff's base-forming reaction of Patel and Lodish (44). Briefly, 5 mg of the peptide and

11.5 mg of bovine serum albumin were dissolved in 1 ml of 0.1 M sodium phosphate, pH 7.5, and to this solution was added dropwise 0.5 ml of 20 mM glutaraldehyde aqueous solution with stirring at 20 °C. The mixture was left for a further 30 min at 20 °C and then the remaining aldehyde groups were blocked with 0.1 M glycine, pH 7.0. The resulting GRGDS-derivatized serum albumin was isolated by chromatography on Sephadex G-100.

Preparation of Glycosaminoglycan-derivatized Serum Albumin—Glycosaminoglycans were coupled to bovine serum albumin by a carbodiimide-promoted condensation reaction. Five mg of glycosaminoglycan and 100 μg of bovine serum albumin were dissolved in 0.5 ml of H_2O, and the solution was adjusted to pH 5.0 with 0.1 M HCl. To this solution was added 25 mg of *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride in 0.5 ml H_2O, pH 5.0, and the mixture was stirred at 4 °C for 12 h. The mixture was centrifugated at 20,000 × *g* for 10 min, and the supernatant solution was collected and exhaustively dialyzed against distilled water. The glycosaminoglycan-derivatized serum albumin was collected by lyophilization.

RESULTS

Inhibition of Cell-Substrate Adhesion by PG-M—When trypsinized BHK21 cells were seeded onto fibronectin-coated dishes, most if not all of the cells adhered to the plate and spread within 2 h. PG-M added simultaneously to the assay medium effectively inhibited cell attachment in a dose-dependent manner (Fig. 1). Under conditions where cell attachment was partially inhibited, the spreading of attached cells was also partially inhibited by PG-M (not shown). The inhibition of cell adhesion by PG-M is not restricted to the BHK21-fibronectin system (Table I). Thus, the fibronectin-mediated adhesion of both mammalian cells (*e.g.* BHK cells and CHO cells) and avian cells (*e.g.* CEF) was similarly inhibited by PG-M, even though the cell types differed with respect to the structure and ligand specificity of their fibronectin receptors (1, 2). In addition, PG-M had strong inhibitory effects on the adhesion of BHK cells to vitronectin or type I collagen (molecules also containing the RGD sequence), the adhesion of BHK, CEF, CHO, and B16F10 cells to laminin (a molecule containing both RGD and YIGSR sequences, which may be recognized by different cell surface receptors, see Refs. 10 and 45–48), and the adhesion of high metastatic B16F10 melanoma cells to fibronectin (in this case, the REDV and CS1 sequences in the type III homology-connecting segment rather than the RGD sequence are most prominant in adhesion, see Ref. 49). Other cell-substrate adhesion systems similarly inhibited by PG-M were: F9 embryonal carcinoma to tissue culture dishes, murine fibroblastic cell line STO cells and primary mouse dermal fibroblasts to fibronectin-coated dishes, and rat pheochromocytoma PC12 cells (in the differentiated state induced by nerve growth factor) to fibronectin- or laminin-coated dishes. In contrast, calcium-dependent cell-cell adhesion of F9 embryonal carcinoma, which is mediated by the cell adhesion molecule E-cadherin (50) or uvomorulin (51), was not affected by PG-M even at 30 μg/ml hexuronate/ml. These results suggest that the proteoglycan acts as an inhibitor of a wide range of cell-substrate adhesion systems, but not on cell-cell adhesion systems and that its action is not strictly dependent either on the cell type or on the substrate adhesion molecules involved.

TABLE I

The inhibitory effect of PG-M on various cell substrate adhesion systems

Cell	Substrate (adhesion molecule)[a]	Relative number of cells attached[b]	
		Control	PG-M added[c]
BHK21	Fibronectin	+++++[d]	—[e]
	Laminin	+	—
	Collagen	+	—
	Vitronectin	++++	—
CEF	Fibronectin	+++++	—
	Laminin	+	—
	Collagen	+	—
	Vitronectin	+++++	—
CHO-K1	Fibronectin	+++++	—
	Laminin	+++++	—
B16F10	Fibronectin	+++++	—
	Laminin	+++	—

[a] Solutions of human plasma fibronectin, mouse EHS tumor laminin, rat tail tendon type I collagen, and bovine serum vitronectin (5 μg/ml) were used for coating the dishes.
[b] Relative numbers of the cells adhering to each plate.
[c] The PG-M concentration = 20 μg hexuronate/ml.
[d] 90–100% cells adhered to the plates.
[e] Very few or no cells adhered to the plate.

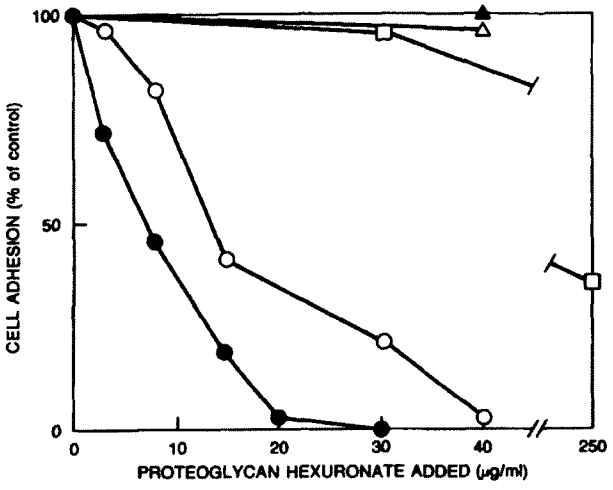

FIG. 1. **Effects of proteoglycans and related compounds on BHK cell adhesion to fibronectin-coated dishes.** Plastic dishes were coated with human plasma fibronectin at the concentration of 5 μg/ml, and trypsinized BHK cells were seeded onto the dishes with medium containing PG-M (●), PG-H (○), or heparan sulfate proteoglycan from the mouse EHS tumor (□) at the concentrations indicated. The effects of PG-M treated with Pronase (▲) or chondroitinase ACII (△) are also shown. All values are the mean of three determinations of percent cell attachment; the range of variation was <10%. 85% seeded cells attached to the control dish (no addition and no treatment).

Structural Requirements for the Inhibitory Activity of PG-M—Besides PG-M, the cartilage-characteristic chondroitin sulfate proteoglycan, PG-H, had strong inhibitory effects on the attachment of BHK cells to fibronectin-coated dishes (Fig. 1) as well as on the other cell-substrate adhesion systems described above, *e.g.* attachment of CHO cells to laminin. In contrast, heparan sulfate proteoglycan form LD derived from the EHS mouse basement membrane tumor had much less of an inhibitory effect (Fig. 1), suggesting that the inhibition is specific with respect to the glycosaminoglycan chains involved.

In order to determine which parts of the chondroitin sulfate proteoglycan molecule are responsible for the inhibitory action, a series of experiments was conducted where the core protein and chondroitin sulfate chains were selectively cleaved with appropriate enzymes, and the resultant fragments were examined for their ability to inhibit the adhesion of BHK cells to fibronectin-coated dishes. Incubation of PG-M in a boiling water bath for 5 min did not alter its inhibitory activity. However, treatment of PG-M with either proteolytic enzymes or chondroitinase ACII followed by denaturation of the enzymes at 100 °C completely abolished the inhibitory

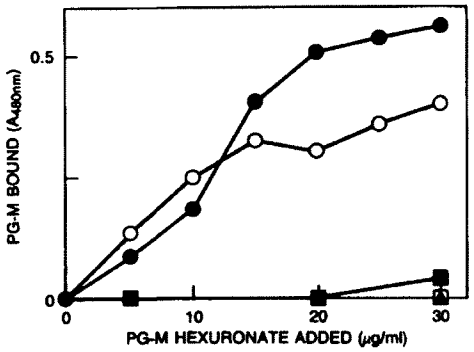

FIG. 2. **Binding of PG-M to fibronectin and its proteolytic fragments.** Plastic wells were coated with solutions of human plasma fibronectin (○), the fibronectin heparin-binding fragment I (▲), collagen-binding fragment (□), cell-binding fragment (■), or heparin-binding fragment II (●), each at a concentration of 10 μg/ml. After incubation with PG-M, bound PG-M was determined by enzyme-linked immunosorbent assay using MO-225, a monoclonal antibody to the chondroitin sulfate chain.

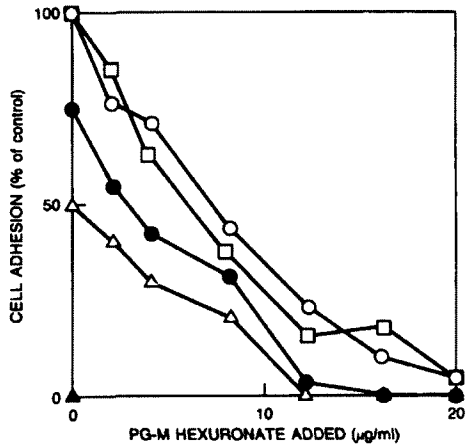

FIG. 3. **Effects of PG-M on BHK cell adhesion to fibronectin and related fragment-coated dishes.** Solutions used for coating the dishes were: human fibronectin, 5 μg (●) or 10 μg (○) per ml; 75,000 cell binding fragment (□), 5 μg/ml; and GRGDS-derivatized albumin, 10 μg/ml (△). Cell adhesion was assayed as in Fig. 1. Heparin-binding fragments (M_r 30,000 and 38,000) and collagen-binding fragment (M_r 29,000) derived from fibronectin did not show any detectable cell adhesion (▲).

activity. A similar abolition of the activity was also observed when PG-H was treated as above (data not shown). Using the same cell-substrate adhesion system, the isolated glycosaminoglycans from PG-M and PG-H, chondroitin sulfate, hyaluronic acid, dermatan sulfate, chondroitin, heparan sulfate, and heparin were shown to have no inhibition activity at 10 mg/ml. Together, the results indicate that neither chondroitin sulfate-free core proteins nor core protein-free chondroitin sulfate chains are capable of inhibiting cell adhesion and that the molecular form consisting of chondroitin sulfate chains attached to a core protein is required for activity.

The Inhibition by PG-M Is Not Due to a Direct Binding of PG-M to Adhesion Molecules—Since PG-M has a moderate capacity to bind to fibronectin (31), the observed inhibition by PG-M could be ascribed to a masking of the cell-binding domain of fibronectin due to direct binding of PG-M to the adhesion molecule, as has been postulated by other investigators who studied the effects of proteoglycans on fibronectin-mediated adhesion (26, 29, 30). Our results, however, indicate that this is unlikely. First, as shown in Table I, the adhesion of cells to laminin, a molecule for which PG-M exhibits no apparent binding activity (31), was efficiently inhibited by PG-M. More direct evidence was obtained in experiments where a M_r 75,000 fragment derived from fibronectin by trypsin digestion was used in place of intact fibronectin. This fragment has been shown to contain the cell-binding domain (39) which consists of two critical synergistic signals for cell adhesion (9). As shown in Fig. 2,[2] this cell-binding domain fragment had no detectable capacity to bind PG-M. As Fig. 3 shows, the adhesion of cells to the M_r 75,000 fragment immobilized on plates was inhibited by PG-M, just as with intact fibronectin. Furthermore, taking advantage of the fact that synthetic GRGDS peptide-conjugated serum albumin can substitute for fibronectin as a substratum for cell adhesion (44), effects of PG-M on the adhesion of BHK cells to GRGDS serum albumin were examined (Fig. 3). Again, cell-substrate adhesion was strongly inhibited by PG-M. Since GRGDS serum albumin had no capacity to bind PG-M (data not shown), direct binding between proteoglycan and fibronectin, if any, cannot account for the inhibition.

Mechanism of PG-M Inhibition of Cell-Substrate Adhesion—After coating with fibronectin (or laminin), the plastic dishes were further incubated with PG-M (or PG-H) at 20 °C for 2 h and subsequently washed with phosphate-buffered saline as described under "Experimental Procedures." In view of the low concentration (5 μg/ml) of fibronectin (or laminin) used in the first coating, some unoccupied protein adsorption sites should remain on the plastic substrate that are available for the PG-M used in the second coating. Using these plates, a series of BHK cell adhesion experiments were carried out to examine whether proteoglycans immobilized on the fibronectin-coated plates could also act as an inhibitor. When the surface of the fibronectin-coated wells was coated with a solution of PG-M (20 μg hexuronate/ml), adhesion was almost completely inhibited. In control experiments, the BHK cells were preincubated at 37 °C for 1 h with a solution of 20 μg (as hexuronate) of PG-M (or PG-H)/ml of assay medium. The cells were then collected by centrifugation, washed with assay medium, and examined for their ability to adhere to a fibronectin substrate with no added proteoglycan. The pretreatment of the cells with PG-M (or PG-H) in medium had no effect on cell adhesion. The results indicate that only the proteoglycan counterpart immobilized on plastic dishes acts as an inhibitor of cell-substrate adhesion. The following observation further supports this view.

When the fibronectin-coated plastic dishes were treated with a high concentration of bovine serum albumin to cover uncoated plastic surfaces, *i.e.* to block all residual protein adsorption sites, subsequent assay with PG-M (added to the medium) indicated that the proteoglycan could no longer inhibit BHK cell adhesion (Fig. 4). Bovine serum albumin itself, when applied to the fibronectin-coated dishes as above, had little inhibitory effect on cell adhesion.

That PG-M, once absorbed into the uncoated or fibronectin-coated plastic dish, was not released by subsequent washing with detergent-containing buffer was confirmed by immunochemical staining of the coated wells with anti-PG-M antibody (Fig. 5). Immunochemical evidence also indicated that PG-M did not bind to dishes pretreated successively with fibronectin and an excess of serum albumin. A detectable

[2] Among the thermolysin-treated fragments, only the M_r 38,000 fragment containing a heparin-binding domain was capable of binding to PG-M (see Fig. 2). Treatment of PG-M with chondroitinase ABC or ACII did not alter its binding activity to the M_r 38,000 fragment, suggesting that the chondroitin sulfate chains of PG-M are not involved in the binding (M. Yamagata, unpublished observation).

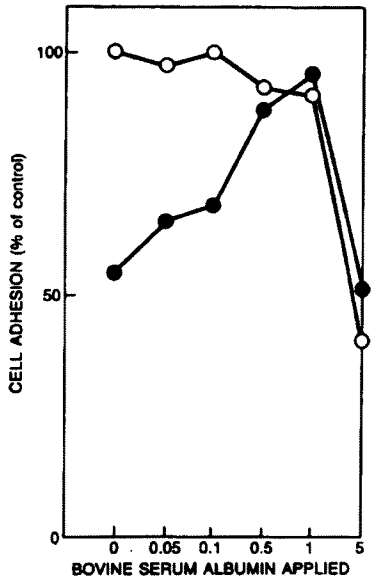

FIG. 4. **Effects of bovine serum albumin and PG-M on BHK cell adhesion to fibronectin-coated dishes.** Plastic plates were coated with 5 μg/ml of human plasma fibronectin and subsequently treated with heat-denatured bovine serum albumin at the indicated concentrations. BHK cells were seeded onto the plates in the persence (●) or absence (○) of 10 μg hexuronate/ml of added PG-M. Each value represents the mean of two determinations.

	A		B	
	Plate	Medium	Plate	Medium
a	— / —	PG-M	— / BSA	PG-M
b	— / —	—	— / BSA	—
c	FN / —	PG-M	FN / BSA	PG-M
d	FN / —	—	FN / BSA	—

I. Cells			II. PG-M			III. Fibronectin		
	A	B		A	B		A	B
a	0	0	a	3	0	a	0	0
b	0	0	b	0	0	b	0	0
c	0	3	c	5	1	c	5	5
d	5	4	d	0	0	d	5	5

FIG. 5. **Schematic presentation of relative numbers of adhering BHK cells (*plate I*), the relative amounts of PG-M (*plate II*), and fibronectin (*plate III*) immobilized onto plastic dishes.** As summarized in the top table, wells were precoated with various combinations of fibronectin, PG-M, and/or albumin as follows. The bottom two rows of wells (*lines c* and *d*) in three plates (*I, II, III*) were treated with a solution of human fibronectin (5 μg/ml), and then the right-hand wells (*lane B*) were treated with 1% (w/v) heat-denatured bovine serum albumin solution. *Plate I*, BHK cells were seeded in the presence (*lines a* and *c*) or absence (*lines b* and *d*) of 20 μg hexuronate of PG-M/ml assay medium, and after incubation, the cells that attached were counted. *Plates II* and *III*, treated as in *Plate I* but BHK cells were omitted. These treated plates were rinsed and then stained with antibodies against PG-M (*Plate II*) or fibronectin (*Plate III*). The values indicate the observed number of attached cells (*Plate I*) or the amount of immobilized PG-M (*Plate II*) or fibronectin (*Plate III*) relative to the maximum number or amount in the respective plate which was taken as 5. *BSA*, bovine serum albumin; *FN*, fibronectin. Values are the mean of two determinations. In *A-d* that showed the maximum cell adhesion, 92% of seeded cells attached to the substrate. No deposition of endogenously synthesized PG-M on the plate was detected at the end of the incubation as shown in *A-b* and *A-d* of *plate II*.

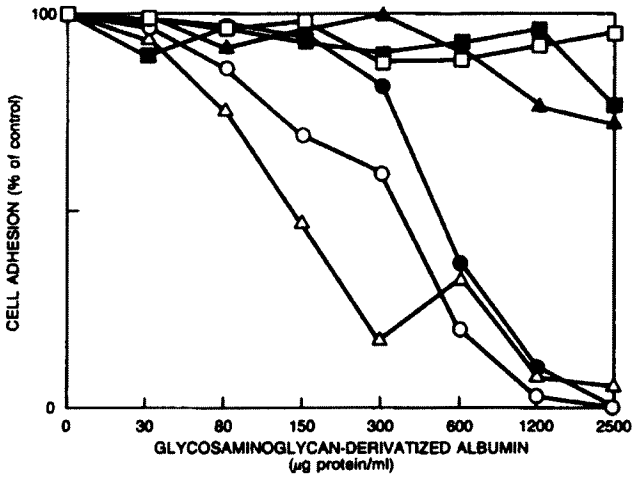

FIG. 6. **Effects on BHK cell adhesion of glycosaminoglycan derivatized serum albumin derivatives coated onto the fibronectin-precoated dish.** After being coated with 5 μg/ml human plasma fibronectin, the plates were coated with glycosaminoglycan-coupled serum albumin at the indicated concentrations. The glycosaminoglycans coupled to bovine serum albumin were: chondroitin sulfate A (●), chondroitin sulfate C (○), chondroitin (□), dermatan sulfate (△), heparan sulfate (▲), or heparin (■). In a control where the plates were similarly treated with a chondroitin sulfate C sample incubated with carbodiimide, no inhibition of cell adhesion was observed (not shown). Bovine serum albumin treated with carbodiimide in the absence of glycosaminoglycans acquired significant cell adhesion activity. Each value represents the mean of two determinations.

amount of PG-M coexisted with immobilized fibronectin itself (presumably by direct binding), but the amount appeared to be much lower than that of the PG-M counterpart bound directly to the plastic surfaces, as demonstrated by the marked differences in total amounts of immobilized PG-M found on the dishes with and without serum albumin treatment (Fig. 5; compare *A-a* and *A-c* with *B-c* in *Plate II*).

More direct evidence for the function of proteoglycans was obtained by cell-substrate adhesion experiments where glycosaminoglycan-conjugated bovine serum albumin derivatives were employed as model proteoglycans. When the serum albumin derivatives were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, both Coomassie Blue and Alcian blue staining revealed a smeared band near the boundary between stacking and separation gels. Treatment of the samples with Pronase resulted in disappearance of the Coomassie Blue-positive band, indicating that the sample indeed contained a protein moiety. As Fig. 6 shows, chondroitin sulfate A (or C)-coupled serum albumin, when immobilized on fibronectin-pretreated dishes, was similar to PG-M in its inhibitory effect on BHK cell-fibronectin adhesion. Free chondroitin sulfate A (or C) chains had no such effects. Since a separate experiment using [^{35}S]sulfate-labeled chondroitin sulfate (prepared from radioactive PG-M) showed that no radioactivity was immobilized on the dishes, the failure of the free polysaccharide chains to inhibit cell adhesion may be ascribed, at least in part, to their inability to attach to the dish. Besides inhibiting BHK cell-fibronectin adhesion, chondroitin sulfate A (or C)-coupled serum albumin inhibited the adhesion of BHK cells to GRGDS albumin complex-coated dishes as well as the adhesion of CHO cells to laminin-coated dishes (data not shown). Interestingly, although dermatan sulfate-coupled serum albumin was likewise inhibitory towards cell-substrate adhesion, chondroitin-, heparin-, or heparan sulfate-coupled serum albumin had far lower inhibitory

activities. The results indicate that the inhibition is specific with respect to the structure of the glycosaminoglycan chain and further suggest that the functional significance of protein moiety is in the immobilization of proteoglycan on the substrate, an essential process for the inhibition, rather than in a direct interaction with cell surface molecules.

DISCUSSION

Although earlier studies (26–30) have demonstrated that the adhesion of fibroblasts to collagen- or fibronectin-coated plates was inhibited by chondroitin sulfate or dermatan sulfate proteoglycans prepared from cartilage or tumors (*i.e.* nonfibroblastic or undefined types of proteoglycans), our finding that the adhesion of trypsinized chick embryo fibroblasts to fibronectin was inhibited by PG-M, the chondroitin sulfate proteoglycan species synthesized by fibroblasts themselves has a more physiological relevance.[3] The *in vitro* function of PG-M we observe may be related to previous findings that the increase in the amount of PG-M (52) correlates with the progression of mesenchymal cell condensation in the developing limb buds of chick embryo. Also related is the observation of Culp *et al.* (53) that detached portions of fibroblasts contain chondroitin sulfate proteoglycan and hyaluronic acid, whereas the attachment sites of fibroblasts contain heparan sulfate proteoglycan and fibronectin. These authors postulated a working model for cell migration in which heparan sulfate proteoglycan and fibronectin play a direct role in cell-substrate adhesion, whereas chondroitin sulfate proteoglycan and hyaluronic acid compete against the adhesion. A functional difference between chondroitin sulfate proteoglycans and heparan sulfate proteoglycans in cell migration was also observed by Kinsella and Wight (54), who reported that heparan sulfate proteoglycan constitutes about 80% of [^{35}S] sulfate-labeled proteoglycan in postconfluent cultures of endothelial cells, whereas after wounding (a procedure to initiate cell migration), chondroitin sulfate/dermatan sulfate proteoglycans[4] increase to 60% of total labeled proteoglycan. Furthermore, the migration of neural crest cells (55) as well as that of cardiac mesenchymal cells (56) were shown to be perturbed by added or coated chondroitin sulfate proteoglycans, suggesting the regulatory role of these proteoglycan in cell migration. These results, coupled with our findings that chondroitin sulfate and dermatan sulfate (but not heparan sulfate) linked to either a core protein or even to serum albumin interfere with cell-substrate adhesion, suggest that chondroitin sulfate/dermatan sulfate proteoglycans synthesized by tissue-forming cells, or more specifically, the PG-M synthesized by fibroblasts are involved in the facilitation of cell migration (after wounding, for example) or cell aggregation (during chondrogenesis, for example) by virtue of their inhibition of cell-substrate adhesion.

Knox *et al.* (28) have reported that cartilage-type chondroitin sulfate proteoglycan does not inhibit the adhesion of BHK cells to a serum preparation containing M_r 70,000 cell-spreading factor (vitronectin). Moreover, Lewandowska *et al.* (30) have reported that a dermatan sulfate proteoglycan prepared from cartilage does not inhibit collagen-mediated adhesion by several types of cells. These reports seemingly contradict the results of our study, in which both cartilage-type chondroitin sulfate and dermatan sulfate-coupled serum albumin can inhibit fibronectin-mediated cell adhesion. This apparent contradiction can be resolved if it is assumed that the serum preparation of Knox *et al.* (28) contained, in addition to vitronectin, some other proteins that interfered with the immobilization of added proteoglycan as has been shown by our experiments with bovine serum albumin (see Fig. 6) and that the amounts of proteoglycan employed by Lewandowska *et al.* (30) were too small to exhibit significant effects.

The precise mechanism for the inhibition of cell-substrate adhesion by PG-M and related substances is not entirely clear at present. Nevertheless, the specificity we find in terms of inhibitory abilities of particular glycosaminoglycan-containing proteoglycans or even of synthetic model proteoglycans, the requirement for their attachment to the substrates, the general effects on several distinct cell-substrate adhesion molecules, plus previous evidence that cell-substrate adhesion depends on specific cell surface receptors, suggest the following scenario by which PG-M or related substances could affect cell-fibronectin interaction. Direct contact of cell surface with chondroitin sulfate/dermatan sulfate chains of proteoglycan immobilized onto the solid phase appears to be a key aspect of the proteoglycan-induced inhibition in substrate-adhesion capacity of the cell. The contact may require a cell surface receptor capable of interacting specifically with chondroitin sulfate/dermatan sulfate chains. The interaction is obviously too weak to induce cell attachment and cell spreading, and it might even be specifically repulsive. The presence of chondroitin sulfate/dermatan sulfate recognition sites on the cell surface must eventually be evaluated. The requirement for immobilization of glycosaminoglycan chains may be due to a necessity that they be a multivalent ligand, which might influence the clustering or conformational change of receptor complexes for fibronectin and other adhesion proteins. Topological interaction between apparently unrelated receptors in the plasma membrane has been implicated in many cellular events including cell-substrate adhesion (57, 58). In relation to the above possibility, the observations by Avnur and Geiger (59) should be noted. They examined the spatial relationship between chondroitin sulfate-containing proteoglycan of the extracellular matrix and cell-substrate focal contacts in chicken gizzard fibroblast cultures using a monoclonal antibody that recognized the chondroitin sulfate moieties of the proteoglycan. The analysis revealed that the substrate-attached chondroitin sulfate proteoglycan was largely depleted from underneath the ends of stress fibers. In the extracellular matrix, components such as fibronectin and proteoglycans may be immobilized onto collagen fibers (or sheets) or as more complex molecular complexes in a solid phase. Thus, the extracellular matrix may affect cell shape, adhesion, and migration by transmitting extracellular positional information to the intracellular cytoskeletal system via transmembrane receptor molecules. It is interesting to note here that some proteoglycans containing chondroitin sulfates occur as constituents of the cell surface membranes (60–62). When the proteoglycans are topologically fixed onto other membrane molecules by its specific associations with them, the chondroitin sulfate chains extracellularly positioned might also affect cell behaviors of neighboring cells through the same processes. At any rate, the results in this communication suggest that endogenous chondroitin sulfate/dermatan sulfate proteoglycans in extracellular matrix have antagonistic effects on a whole host of cell-substrate interactions, thereby potentially modulating a variety of aspects of cell behavior.

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[3] PG-M is not restricted to chick embryo fibroblasts (31) and limb bud (52), but has also been found in the aorta, skin, lung, and muscles of the chick embryo (M. Yamagata, S. Suzuki, and K. Kimata, manuscripts in preparation).

[4] A major proteoglycan species synthesized by endothelial cells has been shown to be very similar to, if not identical with, PG-M (H. Morita, S. Suzuki, and K. Kimata, unpublished data).

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Signal Transduction through the Fibronectin Receptor Induces Collagenase and Stromelysin Gene Expression

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Abstract. We have investigated the effects of ligation of the fibronectin receptor (FnR) on gene expression in rabbit synovial fibroblasts. Monoclonal antibodies to the FnR that block initial adhesion of fibroblasts to fibronectin induced the expression of genes encoding the secreted extracellular matrix-degrading metalloproteinases collagenase and stromelysin. That induction was a direct consequence of interaction with the FnR was shown by the accumulation of mRNA for stromelysin and collagenase. Monoclonal antibodies to several other membrane glycoprotein receptors had no effect on metalloproteinase gene expression. Less than 2 h of treatment of the fibroblasts with anti-FnR in solution was sufficient to trigger the change in gene expression, and induction was blocked by dexamethasone. Unlike other inducers of metalloproteinase expression, including phorbol diesters and growth factors, addition of the anti-FnR in solution to cells adherent to serum-derived adhesion proteins or collagen produced no detectable change in cell shape or actin microfilament organization. Inductive effects were potentiated by cross-linking of the ligand. Fab fragments of anti-FnR were ineffective unless cross-linked or immobilized on the substrate. Adhesion of fibroblasts to native fibronectin did not induce metalloproteinases. However, adhesion to covalently immobilized peptides containing the arg-gly-asp sequence that were derived from fibronectin, varying in size from hexapeptides up to 120 kD, induced collagenase and stromelysin gene expression. This suggests that degradation products of fibronectin are the natural inductive ligands for the FnR. These data demonstrate that signals leading to changes in gene expression are transduced by the FnR, a member of the integrin family of extracellular matrix receptors. The signaling of changes in gene expression by the FnR is distinct from signaling involving cell shape and actin cytoarchitecture. At least two distinct signals are generated: the binding of fibronectin-derived fragments and adhesion-blocking antibodies to the FnR triggers events different from those triggered by binding of the native fibronectin ligand. Because the genes regulated by this integrin are for enzymes that degrade the extracellular matrix, these results suggest that information transduced by the binding of various ligands to integrins may orchestrate the expression of genes regulating cell behavior in the extracellular environment.

THE interactions of cells with components of the extracellular matrix (ECM)[1], such as fibronectin (Fn), laminin (Ln), tenascin, and collagens of more than 12 types, play an important role in morphogenesis, tissue repair and regeneration, and metastasis (Liotta et al., 1986; Chiquet-Ehrismann et al., 1986; Humphries et al., 1986; Gehlsen et al., 1988*a*,*b*). During development and remodeling, cells in tissues constantly alter their morphology, migration, and adhesion to ECM components. Temporal, spatial, and cell type-specific regulation of the expression of this large variety of ECM molecules and their receptors provides a powerful set of mechanisms for generating the diversity required for the proper orchestration of cell behavior during differentiation, morphogenesis, and tissue remodeling.

The integrin multigene family of transmembrane, heterodimeric adhesion receptors mediates cell attachment to a variety of ECM molecules, including Fn, Ln, collagen types I, IV, and VI, vitronectin (Vn), fibrinogen, and thrombospondin (reviewed by Ruoslahti and Pierschbacher, 1986; Buck and Horwitz, 1987). Antibody and peptide inhibition studies have implicated these receptors in processes as di-

Portions of this work have appeared previously in abstract form (Tremble, P. M., C. Damsky, and Z. Werb. 1988. *J. Cell Biol.* 107:150a).

1. *Abbreviations used in this paper*: CM, cell-conditioned medium; DME-LH, serum-free DME supplemented with 0.2% lactalbumin hydrolysate; ECM, extracellular matrix; Fn, fibronectin; FnR, fibronectin receptor; LDL-R, low density lipoprotein receptor; Ln, laminin; RSF, rabbit synovial fibroblasts; TIMP, tissue inhibitor of metalloproteinases; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; Vn, vitronectin.

verse as neurite outgrowth (Bozyczko and Horwitz, 1986; Hall et al., 1987; Tomaselli et al., 1987), gastrulation (Boucaut et al., 1984), neural crest cell migration (Thiery et al., 1985; Bronner-Fraser, 1986), trophoblast outgrowth (Damsky et al., 1985*b*; Richa et al., 1985; Armant et al., 1986; Sutherland et al., 1988), platelet aggregation (Pytela et al., 1986; Phillips et al., 1988), muscle cell attachment to tendons (Bogaert et al., 1987), and tumor cell metastasis (Humphries et al., 1986; Gehlsen et al., 1988*a*).

In addition to establishing a particular set of contacts with the ECM, cells must be able to modify these contacts in a closely regulated fashion. Mechanisms by which this might be accomplished include regulation of the amounts of ligands and receptors present or alteration of ligand-receptor affinity. Proteolysis of ligands or receptors is likely to be involved in the processes of migration, invasion, and tissue repair (Liotta et al., 1986). Inhibition of proteinases inhibits invasion but not attachment of tumor cells (Mignatti et al., 1986; Schultz et al., 1988). Proteinases are present focally at adhesion sites in some cells, actively modulating their actin cytoskeleton and attachments to the substrate (Beckerle et al., 1987; Pöllänen et al., 1988). There is also a strong correlation between changes in the actin cytoskeleton and the induction of expression of collagenase and stromelysin (Aggeler et al., 1984*a*,*b*; Werb et al., 1986), two members of the metalloproteinase gene family (Whitham et al., 1986). Agents that promote cell spreading, such as Fn (Werb and Aggeler, 1978), and agents that inhibit differentiation of some cell types, such as transforming growth factor-β (Edwards et al., 1987), inhibit the phenotypic expression of collagenase. On the other hand, substances that promote cell rounding, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), some proteinases, and cytochalasin B, induce expression of collagenase (Harris et al., 1975; Aggeler et al., 1984*a*,*b*; Werb et al., 1986; Unemori and Werb, 1986, 1988). Taken together, these data suggest that proteinases play a prominent role in regulating adhesion of cells to ECM.

Given the necessity for closely regulating the establishment and modulation of cell–ECM interactions, we used an adhesion-blocking monoclonal antibody against the Fn receptor (FnR) and Fn-derived peptides to explore the possibility that interference with normal cell–Fn interactions can alter gene expression. We show that ligation of the FnR by these ligands, but not by native Fn, induces the expression of metalloproteinases. In contrast to other agents that stimulate expression of these genes, the anti-FnR-mediated induction can occur in the absence of a major change in cell shape or in the reorganization of the actin cytoskeleton. These data suggest that the status of the Fn–FnR interaction is an important signaling mechanism in regulating the expression of genes relevant to matrix remodeling during differentiation.

Materials and Methods

Cells and Cell Culture

Rabbit synovial fibroblasts (RSF) isolated as described previously (Aggeler et al., 1984*a*) and used between passages 1 and 6 were cultured in DME supplemented with 10% FBS. Cells (1–2 × 10^5) were plated in 16-mm wells for 14 h in this medium before washing and replacement with serum-free DME supplemented with 0.2% lactalbumin hydrolysate (DME-LH) for experiments. JAR human choriocarcinoma cells were cultured and maintained as described by Damsky et al. (1985*a*).

Preparation and Characterization of mAbs against Integrins

Whole JAR human choriocarcinoma cells were removed from the culture dishes with 5 mM EDTA, followed by washing with PBS containing Ca^{2+} and Mg^{2+}. A Lewis rat was immunized by a series of fortnightly injections of 10^7 JAR cells, twice intraperitoneally and twice intrasplenically. 4 d after the last injection the immune spleen cells were removed and fused with mouse Sp2/0 plasmacytoma cells and cultured according to the procedure of Kennett (1980) as modified by Wheelock et al. (1987). All wells with growing cells were screened by testing the ability of culture supernatants to inhibit the attachment of JAR cells to Fn or Ln in the attachment assay described by Giancotti et al. (1985) and Tomaselli et al. (1987). Two supernatants inhibited cell attachment to Fn only, whereas two others inhibited cell attachment to both Fn and Ln. After subcloning of the latter, subsequent characterization showed that the two mAbs also inhibited cell attachment to collagen types I and IV. None of the supernatants inhibited attachment to Vn. Detailed characterization of these mAbs will appear elsewhere (Hall, D. E., E. Crowley, and C. H. Damsky, manuscript submitted for publication). One mAb that inhibited cell attachment to Fn only (BIIG2) and one mAb that inhibited cell attachment to Fn, Ln, and the collagens (AIIB2) were selected for further study and designated anti-FnR and anti-β_1, respectively. An unrelated cell-binding rat mAb, BIVF2, of the same subtype was used as a control. The second rat anti-FnR mAb (BIE5) was used in certain experiments where indicated.

The mAbs were further characterized by immunoprecipitation to determine which polypeptides on RSF were recognized by anti-FnR and anti-β_1. Proteins in RSF (~5 × 10^6) were labeled by incubation for 24 h with 50 μCi/ml of [^{3}H]6-D-glucosamine (sp act 25 Ci/mmol; New England Nuclear, Boston, MA) in a low glucose (1 g/liter) formulation of DME. Cells were harvested in 2 mM EDTA, washed, and lysed in 10 mM Tris-acetate buffer, pH 8, containing 0.5% NP-40, 150 mM NaCl, and 2 mM PMSF. Precipitation of integrins by the anti-FnR and anti-β_1 mAbs was carried out according to the procedure described by Tomaselli et al. (1987). Immunoprecipitates were analyzed on 7% SDS-polyacrylamide gels under nonreducing conditions, followed by fluorography.

Preparation of Anti-FnR IgG and Monovalent Fab Fragments

IgG was purified from BIIG2 culture supernatant by anti-rat IgG affinity chromatography with goat anti-rat IgG immobilized on CNBr-activated agarose (Sigma Chemical Co., St. Louis, MO). The IgG was eluted with glycine, pH 2.6, neutralized immediately with Tris base, and dialyzed against PBS, pH 8.0. Affinity-purified BIIG2 in PBS, pH 8.0, was digested with papain (100 mg antibody per 1 mg papain) for 2 h at 37°C (Parham, 1986). The proteins were then alkylated with iodoacetamide (8 mM final concentration); the reaction mixture was dialyzed against 10 mM Tris-buffered saline, pH 8.0, or PBS, pH 8.0; and Fab fragments were separated from Fc fragments and undigested antibody by DEAE-cellulose or protein A-agarose chromatography. IgG and Fab concentrations are expressed in molar concentrations assuming molecular masses of 160 and 50 kD, respectively.

Preparation of ECM Ligand and Antibody Substrates

Culture dishes (Costar, Cambridge, MA; 24- or 48-well plates) were incubated with Fn or Vn at 10 μg/ml in PBS, pH 7.4, overnight at 4°C. Unoccupied sites were blocked with 0.2% bovine serum albumin at ambient temperature for 2 h before cell plating. To assess the extent of collagenase expression on various substrates, cells were plated on Fn, Vn, anti-FnR IgG, or Fab. In some cases these substrates were covalently linked to 12-mm glass coverslips as described below.

Covalent Protein Coating of Glass Coverslips for Specific Adhesion

For covalently linked Fn, type I collagen, peptides containing the arg-gly-asp (RGD) cell recognition sequence, or purified anti-FnR IgG or Fab, polypeptides were conjugated to glass coverslips as follows. Coverslips were washed sequentially with 20% concentrated H_2SO_4, water, 0.1 N NaOH, and water. Dried coverslips were exposed to γ-aminopropyltriethoxysilane (Sigma Chemical Co.) for 4 min at ambient temperature, followed by water and PBS rinses. The coverslips were incubated with 0.25% glutaraldehyde in PBS for 30 min at 22°C, washed several times with PBS, and covered

with ECM proteins or antibody solutions (20–700 μg/ml) for 1 h at ambient temperature, washed with PBS, and used immediately. All steps after the glutaraldehyde treatment were done with sterile reagents and utensils. Freshly trypsinized cells resuspended in DME-LH were plated on the glass coverslips containing immobilized proteins in 24-well plates.

Preparation of mAbs against Collagenase

Rabbit collagenase was purified from cultures of rabbit skin. Fragments of rabbit skin were cultured in DME-LH, and the cell-conditioned medium (CM) was collected every 2 d for up to 16–18 d. Medium from day 4 on was pooled and brought to 25% saturation with $(NH_4)_2SO_4$. The supernatant liquid was dialyzed extensively against 20 mM NH_4HCO_3, freeze-dried, reconstituted in water, and dialyzed against 10 mM Tris-HCl buffer, pH 8.4, with 0.05% Brij-35. Collagenase was purified by DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) chromatography, followed by zinc-chelate affinity chromatography (Cawston and Tyler, 1979; Chin et al., 1985). The collagenase, which was eluted with 50 mM sodium acetate, pH 4.7, was purified 460-fold.

BALB/c mice were immunized with 100 μg of purified collagenase as described by Oi and Herzenberg (1980), except that Freund's complete adjuvant was used for the first injection and Freund's incomplete adjuvant was used for booster injections. The dispersed spleen cells of the mouse were fused with a subclone of the mouse myeloma line P3-X63-Ag8 that does not produce immunoglobulin. The fusion protocol was essentially the same as that described previously (Oi and Herzenberg, 1980), except that warm PBS instead of serum-free medium was added to the polyethylene glycol pellet, and the fused cells were centrifuged at 200 *g* for 8 min and resuspended in 12 ml of RPMI medium containing 15% FBS; 50 μl were placed in each well of a 24-well Costar plate for hybrid selection in medium containing 1×10^{-4} M hypoxanthine, 1×10^{-6} M aminopterin, and 1.6×10^{-5} M thymidine. As the hybrid cells grew, the medium was tested for production of specific antibody by a two-step, solid-phase ELISA, essentially as described by Maggio (1980), with alkaline phosphatase-conjugated anti-mouse IgG as secondary antibody. IgG secretors were subcloned and characterized by immunoblotting and immunoprecipitation of collagenase from TPA-treated RSF and by immunofluorescent staining of TPA-treated RSF. Relevant hybridoma clones were cultured in Ventrex HL-1 serum-free medium (Fisher Scientific Co., Pittsburgh, PA) or injected into BALB/c mice for ascites tumor production of antibody. mAb obtained from both sources was purified over a protein A-Sepharose column (Pharmacia Fine Chemicals). An "oligoclonal" mixture of five anticollagenase mAbs was used in this study.

Biosynthetic Labeling of Proteins Secreted by RSF

RSF proteins were biosynthetically labeled with 25–50 μCi/ml of [^{35}S]methionine (sp act 1,265 Ci/mmol) for 2–4 h in methionine-free DME at 37°C. Proteins were precipitated from the CM with quinine sulfate-SDS and resuspended in Laemmli sample buffer, as previously described (Unemori and Werb, 1986), and samples were analyzed on 10% gels with a 3% stacking gel under reducing conditions. Collagenase and stromelysin were immunoprecipitated from [^{35}S]methionine-labeled secreted proteins with 1–2 μg of monoclonal anti-rabbit collagenase IgG or 10 μg polyclonal sheep anti-stromelysin (Chin et al., 1985), followed by formalin-fixed *Staphylococcus aureus* (Zysorbin; Zymed Labs, Burlingame, CA). Nonimmune mouse IgG (2 μg) or sheep IgG (10 μg) replaced the immune IgG as controls. Total secreted proteins were separated on 7–15% or 10% SDS-polyacrylamide gels and then analyzed by fluorography as described previously (Unemori and Werb, 1986, 1988). All experiments were performed at least twice.

SDS-Substrate Gels for Analysis of Proteinases

CM was subjected to substrate gel electrophoresis in 10% polyacrylamide gels impregnated with 1 mg/ml gelatin or casein (Unemori and Werb, 1986; Herron et al., 1986). Unconcentrated CM was mixed with Laemmli sample buffer (lacking β-mercaptoethanol and modified to contain a final concentration of 2.5% SDS) and electrophoresed under nonreducing conditions. After electrophoresis, the gel was incubated in 2% Triton X-100 for 30 min at 37°C to remove SDS and then incubated for 18–24 h at 37°C in substrate buffer (50 mM Tris-HCl buffer, pH 8, containing 5 mM $CaCl_2$). After staining with Coomassie blue R250, gelatin- or casein-degrading enzymes present in the CM were identified as clear zones in a blue background.

RNA Isolation, Blotting, and Hybridization

Total RNA was isolated from cultured RSF by the method of either Chirgwin et al. (1979) or Cathala et al. (1983). RNA was separated on agarose gels, transferred to nylon membranes, and probed with ^{32}P-labeled cDNA probes as previously described (Maniatis et al., 1982; Frisch et al., 1987). cDNA clones for rabbit collagenase (pCL1), stromelysin (pSL2; Frisch et al., 1987), human tissue inhibitor of metalloproteinases (TIMP) (SP65 TIMP/erythroid-potentiating activity; sequence identical to that described by Docherty et al., 1985; gift of M. Wrann, Sandoz Research Laboratories, Vienna, Austria), human 68-kD gelatinase/type IV collagenase (Collier et al., 1988; gift of G. Goldberg, Washington University, St. Louis, MO), and human γ-actin (Engel et al., 1981; gift of L. Kedes, Stanford University, Stanford, CA) were used to generate probes.

Colocalization of Collagenase and Actin Microfilament Bundles in RSF

Cells were plated in 24-well plates (Costar) in DME containing 10% FBS on 11-mm round glass coverslips for 8 h to permit spreading. Medium was then replaced with DME-LH. Anti-FnR IgG or control IgG was added at a concentration of 15 μg/ml. At various times, medium containing IgG was removed and replaced with DME-LH. At 24 h, coverslips were rinsed with DME-LH and fixed for 10 min in 3% paraformaldehyde in PBS containing 0.5 mM Ca^{2+} and then stained by a modification of the protocol of Damsky et al. (1985*a*). Briefly, after fixation, cells on coverslips were rinsed, made permeable with acetone at 4°C for 3 min, and incubated with PBS containing 0.2% bovine serum albumin. Cells were exposed for 1 h to a cocktail of mouse monoclonal antibodies against rabbit collagenase (Unemori and Werb, 1988). Cells were rinsed and exposed for 1 h to biotinylated goat anti-mouse IgG (Sigma Chemical Co.), rinsed again, and exposed for 30 min to a mixture of 1:100 dilution of FITC-labeled streptavidin (Amersham Corp., Arlington Heights, IL) and 1 μg/ml of rhodamine-phalloidin (Molecular Probes, Inc., Junction City, OR). The washed coverslips were then mounted in Gelvatol containing phenylenediamine to reduce quenching of the fluorescein signal (Platt and Michael, 1983) and examined with a phase epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with filters suitable for FITC and rhodamine and a 63× Planapo oil immersion objective. Cells were photographed with Eastman Kodak Co. (Rochester, NY) Tri-X film and developed with Accufine.

Other Antibodies and Reagents

A mouse anti-FnR mAb (gift of D. Cheresh, Scripps Clinic and Research Foundation, La Jolla, CA) was analyzed in certain experiments where indicated. A mouse mAb binding to the rabbit low density lipoprotein receptor (LDL-R) (gift of T. Innerarity, Gladstone Foundation Laboratories, University of California, San Francisco, CA) and a mouse mAb to the transferrin receptor (AMAC Inc., Westport, ME) were used as controls for antibodies binding to other membrane receptors. Gly-arg-gly-asp-ser-pro (GRGDSP) and gly-arg-gly-glu-ser-pro (GRGESP) peptides were gifts of E. Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA) or were purchased from Telios, Inc. (La Jolla, CA). Gly-D-arg-gly-asp-ser-pro-ala-ser-ser-lys (GdRGDSPASSK), gly-arg-gly-asp-asn-pro (GRGDNP), and the 120-kD and 60-kD cell-binding fragments of Fn were purchased from Telios, Inc. Vn was a gift of Dr. M. Pierschbacher (La Jolla Cancer Research Foundation, La Jolla, CA); anti-Fn antibody, Ln, and Fn were purchased from Collaborative Research Inc., Waltham, MA. Type I collagen (Vitrogen) was purchased from Collagen Corp. (Palo Alto, CA).

Results

An mAb Recognizes the FnR in Rabbit Fibroblasts

We first tested an mAb to the FnR (BIIG2), one of the β_1 family of integrins, that was produced from a rat immunized with JAR human choriocarcinoma cells. Anti-FnR interfered with the initial attachment of many human cell types (data not shown), as well as RSF, to Fn (Fig. 1 *A*), but not to Ln, collagen types I and IV, or Vn (data not shown). Anti-FnR immunoprecipitated a dimer migrating at 110 and 150 kD under nonreducing conditions from NP-40 extracts of [^{3}H]glucosamine-labeled RSF (Fig. 1 *B*). We then used another mAb, anti-β_1, which recognizes the common β-chain of the β_1-integrins, to determine which other members of this subfamily are present in RSF. Anti-β_1 interfered with

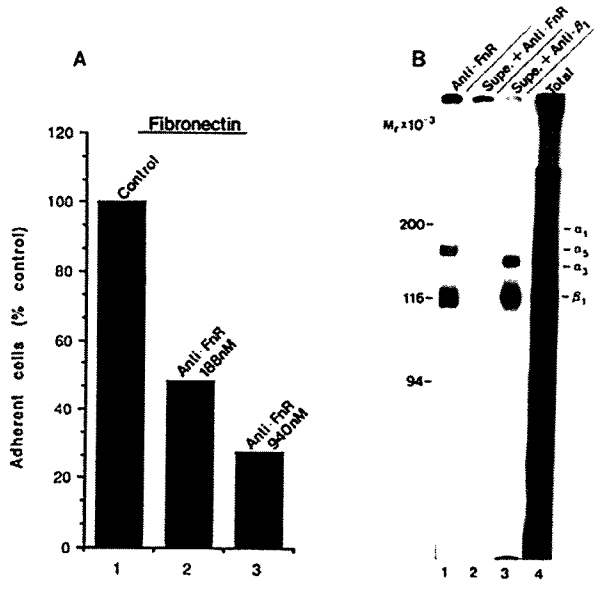


Figure 1. Characterization of an mAb that recognizes the FnR. (*A*) Inhibition of initial adhesion of RSF on culture wells coated with 10 μg/ml of Fn by anti-FnR (BIIG2) at 0.43 or 1.1 μM compared to the control mAb (BIVF2) at 0.63 μM. (*B*) Immunoprecipitation of integrins from NP-40 lysates of [^{3}H]glucosamine-labeled RSF. The immunoprecipitates were separated by SDS-PAGE under nonreducing conditions. The lysate was immunoprecipitated with anti-FnR (lane *1*), and the supernatant (*Supe*) was immunoprecipitated sequentially with anti-FnR (lane *2*) and anti-β_1 (lane *3*); total lysate is shown in lane *4*. The molecular weight standards ($\times 10^{-3}$) and the migration of the FnR α-chain (α_5), VLA_1 α-chain (α_1), VLA_3 α-chain (α_3), and β_1-chain are indicated.

attachment of RSF and several human cell types to Fn, Ln, and collagen types I and IV, but not to Vn, a member of the β_3-subfamily of integrins (data not shown). Anti-β_1 immunoprecipitated the 110-kD band of the β_1-chain and two α-chains at 140 and 190 kD that are distinct from the FnR α-chain (Fig. 1 *B*). Sequential depletion experiments (data not shown) indicated that these α-chains correspond to the integrins VLA_1 and VLA_3 (Takada et al., 1987). Thus, RSF express three members of the β_1-family of integrins, including the FnR (VLA_5).

Anti-FnR Induces Expression of Collagenase and Stromelysin

Alteration of cell adhesion and shape by proteinases such as trypsin (Werb and Aggeler, 1978), culture on surfaces of varying adhesivity (Aggeler et al., 1984*b*), and collagen gel tension (Unemori and Werb, 1986) results in a change in gene expression in fibroblasts characterized by synthesis and secretion of the metalloproteinases collagenase and stromelysin. It was therefore of interest to determine whether perturbation of the FnR would affect collagenase and stromelysin gene expression. Accordingly, we cultured RSF for 24 h in uncoated tissue culture wells in medium containing serum and then treated them with anti-FnR or an unrelated rat mAb as a control. After treatment of RSF with anti-FnR, a striking induction of several secreted proteinases was observed by zymography of the CM in SDS-substrate gels containing gelatin (Fig. 2 *A*) or casein (Fig. 2 *B*). In particular, bands corresponding to the proenzymes of the metalloproteinases collagenase (Fig. 2 *A*) and stromelysin (Fig. 2 *B*) were visible. The induction of collagenase and stromelysin activity by anti-FnR was selective, because the expression of several other metalloproteinases, including the 68-kD gelatinase/type IV collagenase, was unchanged by the treatment.

Analysis of newly synthesized secreted proteins showed that polypeptides migrating at 51, 53, and 57 kD were induced in a concentration-dependent fashion by anti-FnR but not by control mAb (Fig. 3 *A*). mAbs recognizing two other membrane glycoprotein receptors, the LDL-R and the transferrin receptor, did not induce these polypeptides at concentrations of up to 625 nM (data not shown). Treatment of RSF with <25 nM (4 μg/ml) anti-FnR was effective in inducing expression of these polypeptides. Immunoprecipitation with specific antibodies indicated that the 53- and 57-kD bands were procollagenase and the 51-kD band was prostromelysin (Fig. 3 *B*). After treatment with anti-FnR, the two proen-

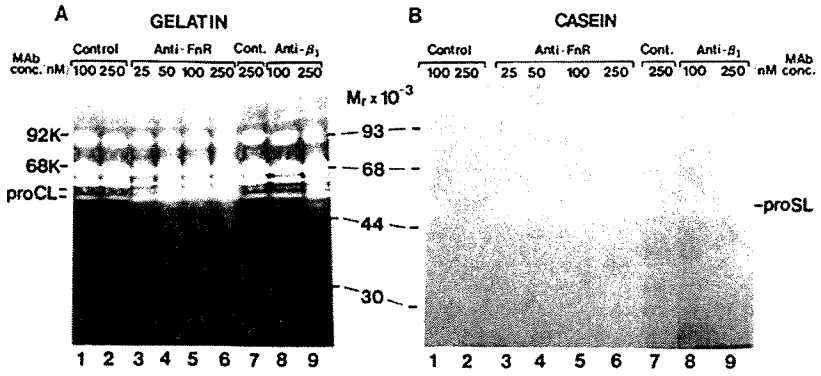


Figure 2. SDS-substrate gel zymography of secreted metalloproteinases induced by treating RSF with anti-FnR. RSF cultured in 48-well plates for 24 h in DME supplemented with 10% FBS were treated with various concentrations of control mAb (lanes *1*, *2*, and *7*), anti-FnR (lanes *3–6*), or anti-β_1 (lanes *8* and *9*) as culture supernatants in DME-LH for 16 h. The cultures were then washed and incubated in DME-LH for 24 h. Samples (10 μl) of the CM were then separated on SDS-substrate gels containing either gelatin (*A*) or casein (*B*), and the zymograms were developed. The migration of prestained molecular weight standards and the bands corresponding to procollagenase (*proCL*), prostromelysin (*proSL*), 68-kD gelatinase (*68K*), and 92-kD gelatinase (*92K*) (Unemori and Werb, 1988) are indicated.

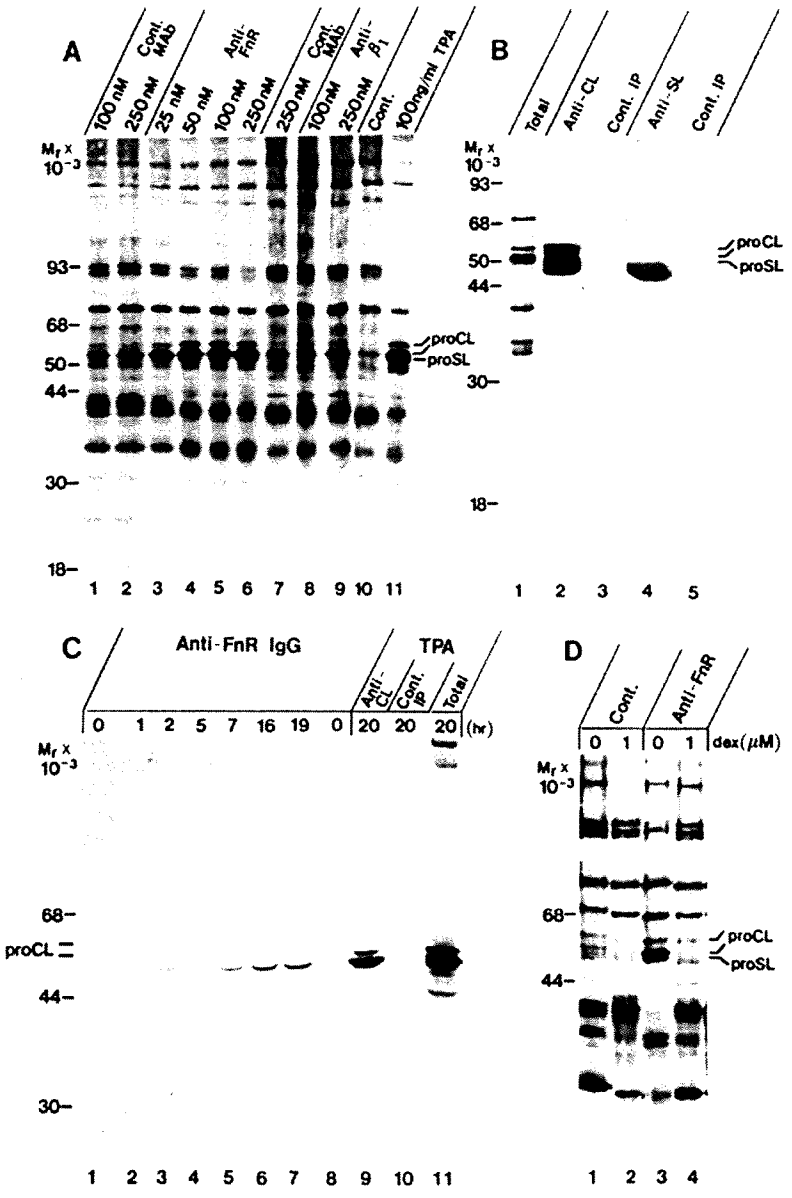


Figure 3. Expression of collagenase and stromelysin gene products by RSF treated with anti-FnR mAb. (*A*) RSF were treated with various concentrations of control mAb (lanes *1*, *2*, and *7*), anti-FnR (lanes *3–6*), or anti-β_1 (lanes *8* and *9*) as culture supernatants, or TPA (lanes *10* and *11*) as described in the legend to Fig. 2, then biosynthetically labeled with [^{35}S]methionine for 4 h. The secreted proteins were analyzed by SDS-PAGE followed by fluorography. (*B*) Collagenase and stromelysin bands were identified by immunoprecipitation of labeled secreted proteins from treated RSF. Total secreted proteins in 50 μl of CM are shown in lane *1*. Proteins were immunoprecipitated from 400 μl of CM with anticollagenase IgG (lane *2*), nonimmune mouse IgG (lane *3*), antistromelysin IgG (lane *4*), or nonimmune sheep IgG (lane *5*), and analyzed by SDS-PAGE followed by fluorography. (*C*) Time course of induction of collagenase secretion by anti-FnR. Confluent RSF were incubated with 310 nM anti-FnR in DME-LH for 1–19 h and then anti-FnR was removed and the cells were cultured in DME-LH for the remaining time up to a total time of 20 h (lanes *1–8*). The cells were then labeled with [^{35}S]methionine for 4 h. As a positive control, RSF were treated with TPA for 20 h (lane *9*) before labeling. The CM containing the biosynthetically labeled proteins was collected, and collagenase was immunoprecipitated with anticollagenase and separated by SDS-PAGE. Lane *10* shows immunoprecipitation with control mouse mAb, and lane *11* shows the total secreted proteins of TPA-treated cells. (*D*) Effect of dexamethasone on induction of collagenase by anti-FnR. RSF were untreated (lane *1*), treated with 1 μM dexamethasone (lane *2*), or treated with anti-FnR as described in *A*, with (lane *4*) or without (lane *3*) 1 μM dexamethasone before biosynthetic labeling with [^{35}S]methionine and separation of secreted proteins by SDS-PAGE. Molecular weight standards ($\times 10^{-3}$) and the migration of procollagenase (*proCL*) and prostromelysin (*proSL*) are indicated.

zymes accounted for as much as 5% of the total secreted proteins of RSF. Less than 2 h of exposure to anti-FnR was required for collagenase expression to be evident at the 24-h evaluation point (Fig. 3 *C*). Anti-β_1 also induced collagenase and stromelysin but was less effective than anti-FnR (Fig. 3 *A*). Two human fibroblast lines (MRC-5 and WI-38) also responded to treatment with the anti-FnR by induced expression of collagenase (data not shown). Two additional adhesion-blocking anti-FnR antibodies, the rat mAb (BIE5) and a mouse mAb, were qualitatively similar to the BIIG2 anti-FnR mAb in inducing collagenase and stromelysin expression in RSF (data not shown). In addition, dexamethasone suppressed the expression of the metalloproteinases induced by anti-FnR (Fig. 3 *D*). In the following sections we concentrate on collagenase expression, although similar induction was generally seen for stromelysin.

We next used RNA blotting analysis to identify collagenase and stromelysin transcripts in RNA extracted from RSF treated with anti-FnR. Untreated RSF contained little mRNA for either metalloproteinase. Treatment of RSF with anti-FnR induced expression of mRNA for collagenase and stromelysin coordinately (Fig. 4) as in treatment with TPA (Frisch et al., 1987; Unemori and Werb, 1988) but in lower amounts (data not shown). In contrast, the expression of mRNA transcripts of the 68-kD gelatinase/type IV collagenase (Collier et al., 1988) and actin was constitutive and was not affected by treatment with anti-FnR (Fig. 4). The mRNA for TIMP decreased very slightly in response to anti-FnR, whereas it

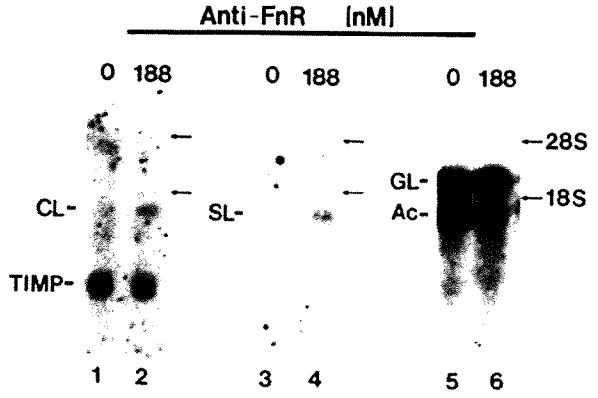


Figure 4. Regulation of metalloproteinase mRNA expression in RSF by anti-FnR. Blotting analysis of RNA isolated from untreated RSF (lanes *1*, *3*, and *5*) or RSF treated with 188 nM anti-FnR (lanes *2*, *4*, and *6*) for 14 h followed by 14 h in DME-LH alone. Total RNA (10 μg) was separated on agarose gels, transferred to nylon membranes, and then hybridized with ^{32}P-labeled inserts from cDNAs encoding collagenase (*CL*) and TIMP (lanes *1* and *2*), stromelysin (*SL*) (lanes *3* and *4*), or 68-kD gelatinase (*GL*) and actin (*Ac*) (lanes *5* and *6*). Migration of 28-S and 18-S rRNA are also indicated.

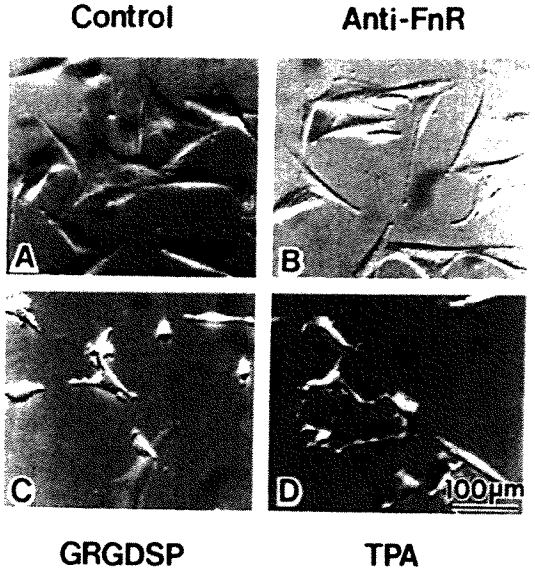


Figure 5. Low power morphological appearance of RSF treated with anti-FnR or a peptide containing RGD. RSF were treated with (*A*) control mAb (250 nM), (*B*) anti-FnR (250 nM), (*C*) GRGDSP (100 μg/ml), or (*D*) TPA (50 ng/ml) for 24 h. Phase-contrast microscopy. Untreated RSF and RSF treated with GRGESP (not shown) were indistinguishable from RSF treated with control mAb in *A*.

is induced by TPA (Murphy et al., 1985; Herron et al., 1986; Unemori and Werb, 1986).

Anti-FnR Induces Collagenase Expression in the Absence of Changes in Cell Shape

Previous experiments using TPA, cytochalasin D, calcium ionophore, collagen gel contraction, and poorly adhesive substrates indicated that induction of collagenase is strongly correlated with cell rounding and a substantial reorganization of the actin cytoskeleton, as determined by staining of actin filaments with rhodamine-phalloidin (Aggeler et al., 1984*a*,*b*; Unemori and Werb, 1986, 1988; Werb et al., 1986). When RSF were plated in the presence of serum that contained at least two adhesion proteins (Vn, Fn), they showed both a marked shape change (Fig. 5) and induction of collagenase expression (Fig. 6) after treatment with TPA. Under the same conditions, however, anti-FnR induced collagenase expression in the absence of apparent cytoskeletal reorganization: RSF maintained both a flattened morphology and elaborate arrays of rhodamine-phalloidin-staining actin microfilament bundles throughout the induction period (Fig. 5), and yet the cells went on to express collagenase (Fig. 6). These results are summarized in Table I. The adherent area of the anti-FnR mAb-treated cells differed from control RSF by <5% (data not shown).

Further evidence that shape change can be divorced from collagenase induction is indicated by the observations on cells plated and spread on immobilized anti-FnR as the substrate. Under these conditions, the cells displayed a flattened morphology but expressed collagenase (Table I; Fig. 7 *A*). Cells spread on type I collagen, Fn, or anti-LDL-R immobilized by the same procedure did not express collagenase. Therefore, immobilized anti-FnR acts as an inductive substrate, whereas several other substrates including Fn, the natural ligand of the FnR, do not.

To explore the relationship between cell shape and metalloproteinase gene expression further, anti-FnR mAb was added to RSF cultured on covalently immobilized purified ECM substrates. Anti-FnR induced collagenase expression by RSF cultured on type I collagen, Fn (Fig. 7 *A*), and Vn

Table I. Lack of Correlation between Induction of Collagenase and Stromelysin Gene Expression and Changes in Cell Shape

Inducing agent	Cell substrate	Cell shape	Collagenase/ stromelysin induced*
None	Serum	Flat	No
None	Collagen	Flat	No
None	Fn	Flat	No
None	Anti-LDL-R	Flat	No
TPA	Serum	Rounded	Yes
Cytochalasin‡	Serum	Rounded	Yes
Collagen gel contraction‡	Serum	Rounded	Yes
Proteinases‡	Serum	Rounded	Yes
Soluble anti-FnR	Serum	Flat	Yes
Soluble anti-FnR	Fn	Rounded	Yes
Soluble anti-FnR	Collagen	Flat	Yes
Immobilized anti-FnR	Anti-FnR	Flat	Yes
GRGESP	Serum	Flat	No
GRGDSP	Serum	Rounded	Yes
Immobilized anti-FnR plus soluble GRGDSP	Anti-FnR	Rounded	Yes
GRGDSP	Collagen	Flat	No
Immobilized GRGDSP	GRGDSP	Flat	Yes
Immobilized Fn peptides	Fn peptides	Flat	Yes

* Induction of proteinases was determined by incorporation of [^{35}S]methionine into newly synthesized secreted proteins and/or by immunocytochemistry.
‡ Data are from Unemori and Werb (1986); Werb and Aggeler (1978); and Werb et al. (1986).

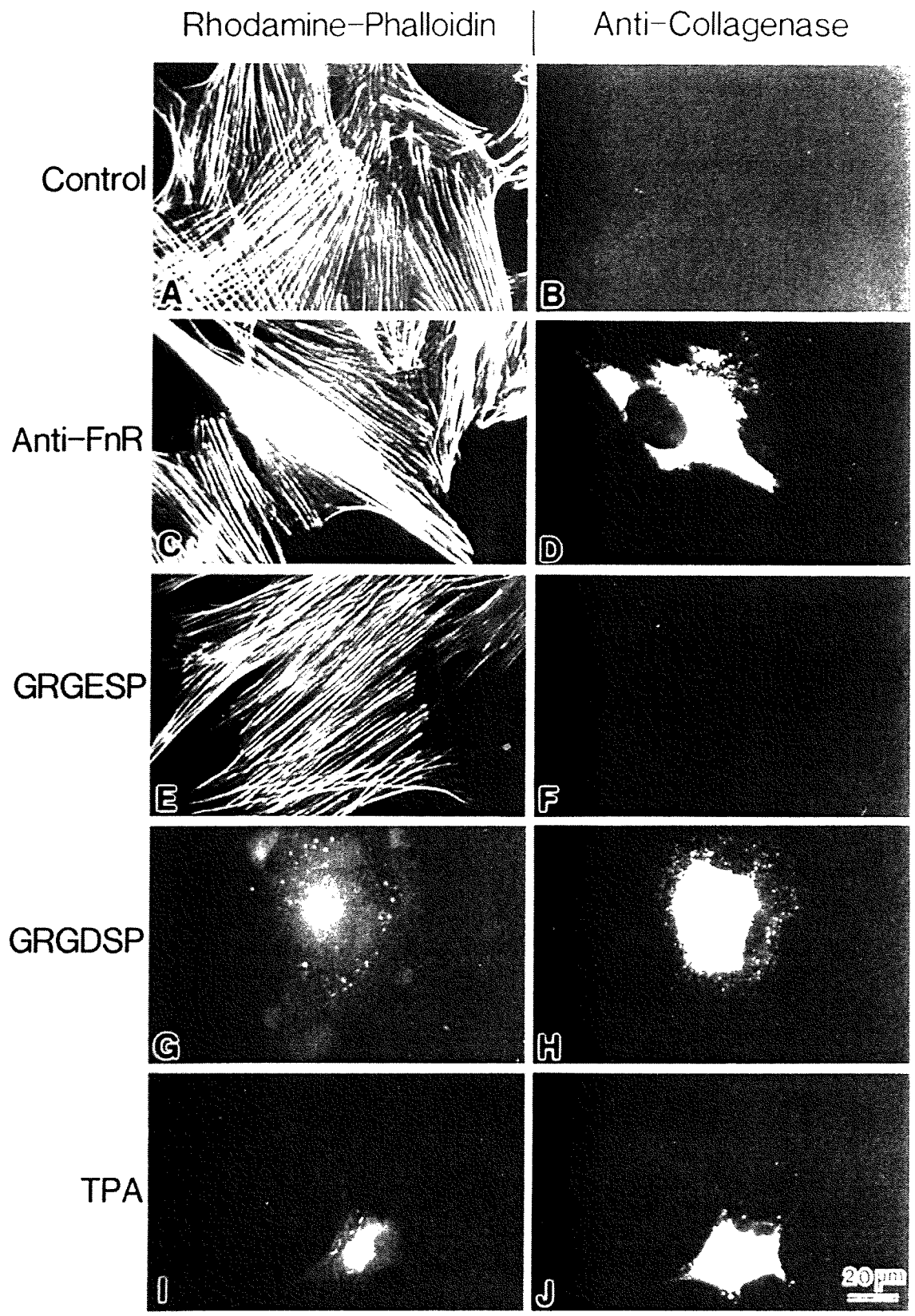


Figure 6. Localization of collagenase and actin microfilaments in RSF treated with anti-FnR or a peptide containing RGD. RSF plated on glass coverslips were left untreated (*A* and *B*) or treated with (*C* and *D*) anti-FnR (250 nM), (*E* and *F*) GRGESP (100 μg/ml), (*G* and *H*) GRGDSP (100 μg/ml), or (*I* and *J*) TPA (50 ng/ml), fixed, and double stained with (*left*) rhodamine-phalloidin to visualize actin microfilaments and (*right*) anticollagenase mAb followed by biotinylated anti-mouse IgG and fluorescein-streptavidin to visualize intracellular collagenase. Paired fluorescence micrographs are shown.

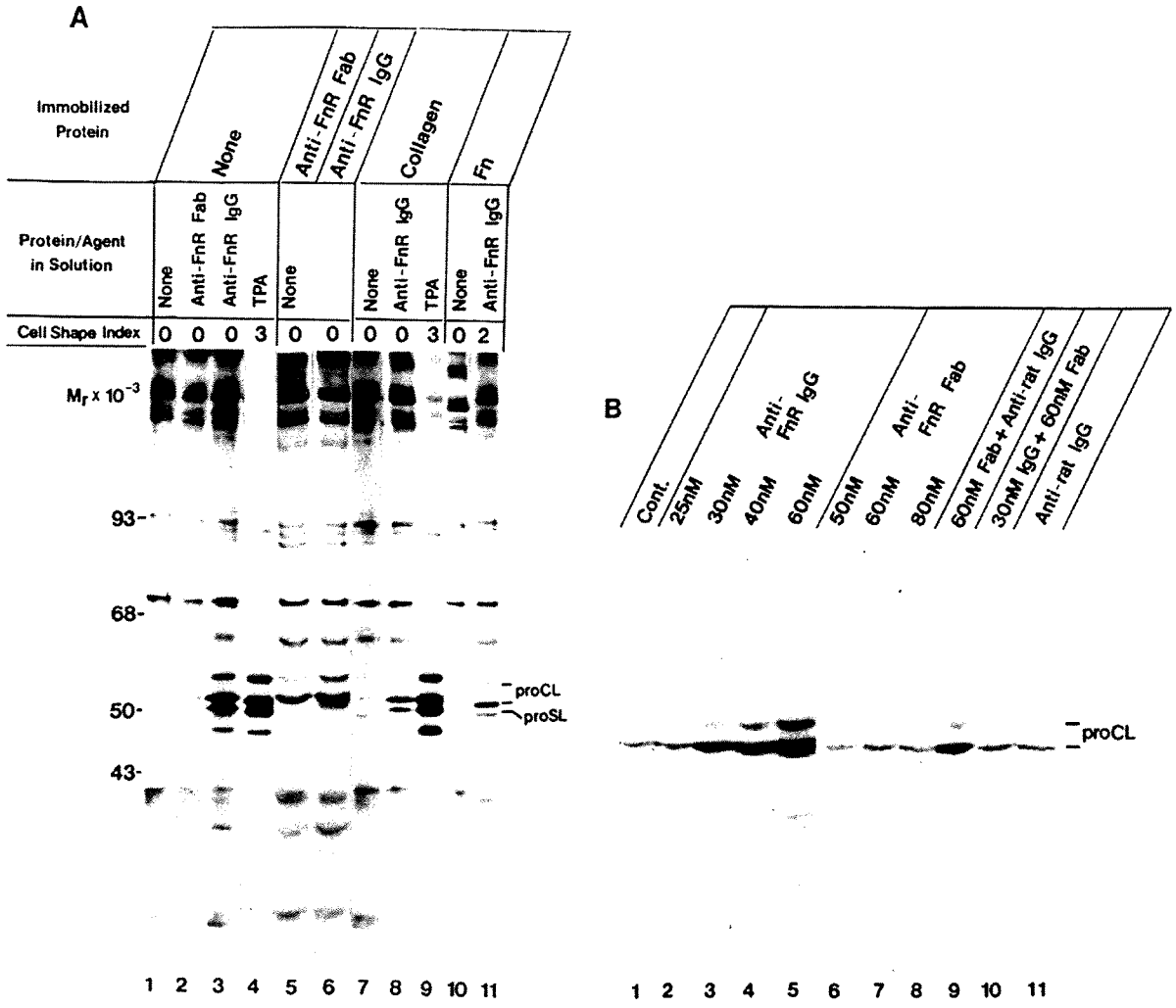


Figure 7. Induction of metalloproteinase expression by immobilized anti-FnR and requirement for cross-linked anti-FnR mAb. (*A*) RSF were plated on uncoated coverslips (lanes *1–4*) in DME supplemented with 10% FBS for 4 h, then placed in DME-LH, or on coverslips coated with covalently immobilized anti-FnR Fab (lane *5*), anti-FnR IgG (lane *6*), type I collagen (lanes *7–9*), or Fn (lanes *10* and *11*) in DME-LH. Soluble anti-FnR Fab at 400 nM (lane *2*), anti-FnR IgG at 115 nM (lanes *3*, *8*, and *11*), or TPA at 100 ng/ml (lanes *4* and *9*) was added. After 20 h the medium was removed and cultures were incubated with [^{35}S]methionine for 4 h to label proteins. The labeled secreted proteins were analyzed by SDS-PAGE followed by fluorography. The cell shape of the treated RSF, rated on a scale of 0–4, indicating flat to round but still attached (Aggeler et al., 1984*b*), is indicated across the top of the gel. The data are from two different experiments. (*B*) To examine the requirement for cross-linking, confluent RSF in 48-well plates were left untreated (lane *1*) or treated with 25–60 nM anti-FnR IgG (lanes *2–5*), 50–80 nM anti-FnR Fab (lanes *6–8*), 60 nM anti-FnR Fab plus 80 μg/ml rabbit anti-rat IgG (lane *9*), or 30 nM anti-FnR IgG plus 60 nM anti-FnR Fab (lane *10*). Control rabbit anti-rat IgG (80 μg/ml) alone is shown in lane *11*. The migration of procollagenase (*proCL*) is indicated.

(data not shown) but changed the cell shape only in cells cultured on Fn. Antibody to the Fn substrate itself also rounded up cells cultured on Fn and induced expression of collagenase in these cells (Table I).

Cross-linking Potentiates Induction of Collagenase Expression by Anti-FnR

The bivalent nature of antigen–antibody interactions suggests that cross-linking of the FnR in the plane of the membrane by anti-FnR may be important to the induction process. To evaluate this possibility, RSF were treated with monovalent anti-FnR Fab or bivalent anti-FnR IgG. Even at twice the equimolar concentration, monovalent anti-FnR Fab was much less effective than bivalent anti-FnR IgG in inducing expression of metalloproteinase, and competing anti-FnR Fab reduced the inducing effect of anti-FnR IgG (Fig. 7 *B*). Inducing activity of the anti-FnR Fab was increased when a secondary anti-rat IgG was added to cross-link the FnR artificially by interacting in a bivalent manner with the anti-FnR Fab.

A second line of evidence that cross-linking of the FnR by anti-FnR is important comes from the observation that immobilization of monovalent anti-FnR Fab on the substrate enhanced its collagenase-inducing activity (Fig. 7 *A*). These data suggest that aggregation of the FnR by anti-FnR, and not just occupancy by the anti-FnR mAb, is important in the gene induction events (Table I).

Fibronectin-derived Peptides Induce Collagenase and Stromelysin Expression

Although antibodies to receptors may behave as high affinity ligands, the anti-FnR mAb did not mimic the effects of native Fn. Therefore, we sought another physiological ligand with the same effector functions as the antibody. Hexapeptides containing RGD, which is present in Fn, Vn, and other ECM ligands that interact with integrins, interfere with cell adhesion (Pytela et al., 1986; Pierschbacher and Ruoslahti, 1987) and differentiation events (Menko and Boettiger, 1987). If the effects of anti-FnR on metalloproteinase gene expression are due to interference with the interaction of RSF with Fn, then RGD peptides should also induce metalloproteinases. We found that the GRGDSP peptide, but not the control GRGESP peptide, when added to RSF cultured in the presence of serum, induced a concentration-dependent increase in collagenase and stromelysin synthesis and secretion, as analyzed by zymography (Fig. 8 *A*) and by biosynthetic labeling of newly synthesized secreted proteins (Fig. 8 *B*). However, unlike the treatment with anti-FnR mAb, treatment with the GRGDSP peptide caused a marked shape change and reorganization of actin microfilament bundles along with the induction of collagenase expression (Figs. 5, 6, and 8, *A* and *B*). The GRGESP peptide produced no shape change, actin rearrangement, or collagenase induction.

Because the GRGDSP sequence is not recognized by the collagen receptor of the integrin class, binding to collagen is not reversed by this peptide (Dedhar et al., 1987). In contrast to cells plated in serum, when the GRGDSP peptide was added to RSF plated on a collagen substrate the cells did not round up, and metalloproteinase gene expression was not induced (Table I). These results were surprising because they suggested that the RGD peptide induces metalloproteinase gene expression by a mechanism dependent on a change in cytoarchitecture rather than the shape-independent mechanism induced by the anti-FnR. On the other hand, the GRGDSP peptide caused cell rounding in the RSF plated on immobilized anti-FnR (Table I), suggesting that anti-FnR recognizes a site on the FnR close to that bound by the RGD cell-recognition sequence on Fn.

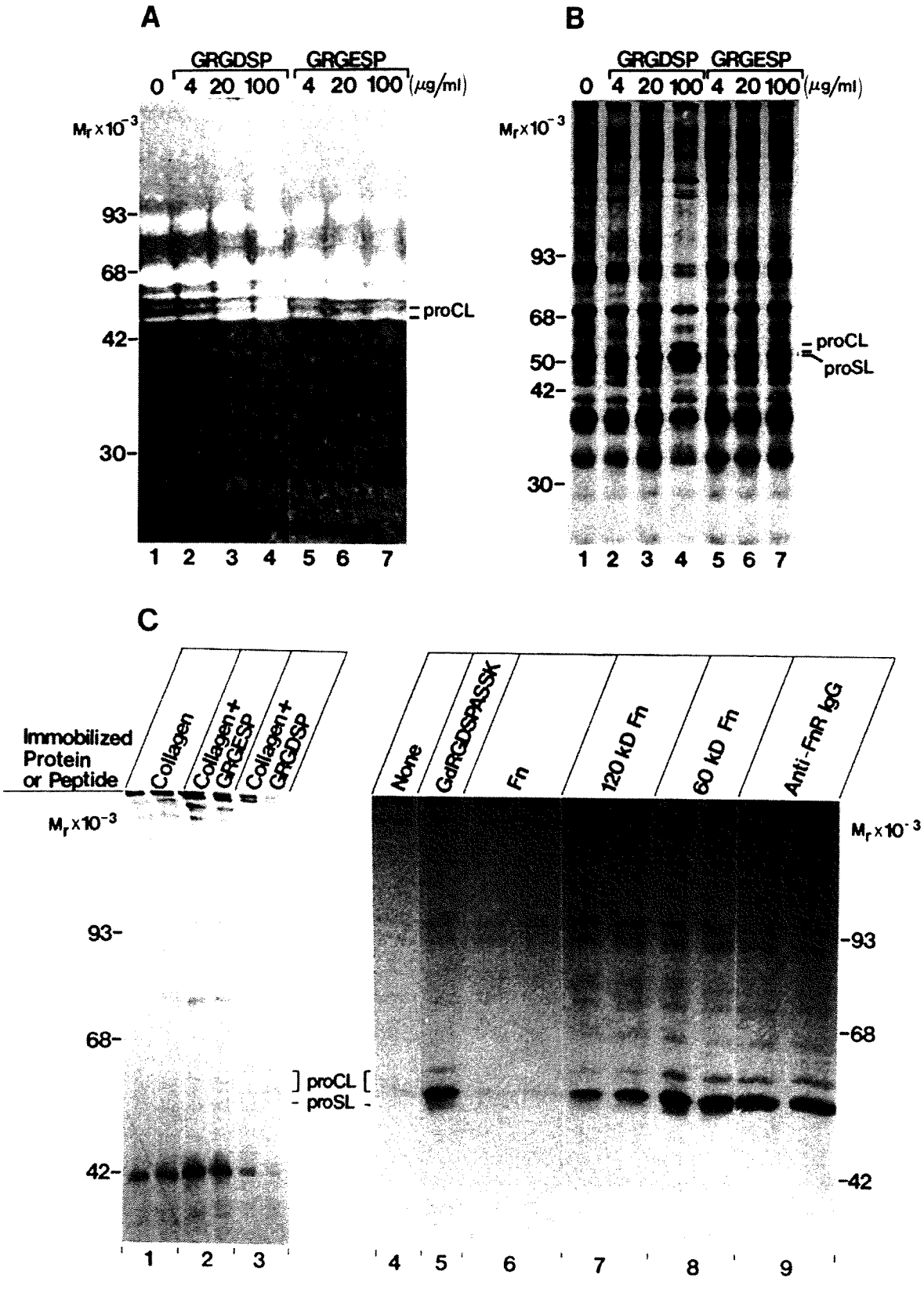
These disparate observations can be reconciled with the results with the anti-FnR mAb when we consider that the RGD peptide is a monovalent ligand. If the RGD peptide behaves as the natural inductive ligand for the FnR receptor only when it is present in a multimeric form, then immobilization of the peptide on the substrate should potentiate its inductive effect. RSF spread on immobilized GRGDSP, but not on GRGESP, rendering GRGESP inappropriate as a control. However, when GRGDSP or GRGESP was immobilized by cross-linking in the presence of collagen, RSF spread and assembled an organized actin cytoskeleton on both substrates. Collagenase expression was induced only in the cells spread on the immobilized GRGDSP, not on GRGESP or collagen alone (Fig. 8 *C*). Soluble, monovalent GRGDSP had no effect on RSF plated on collagen (Table I). Because GRGDSP is recognized not only by the FnR but also by the Vn receptor and other integrins, it has less selectivity than does the anti-FnR mAb. Therefore, we tested two other peptides, GdRGDSPASSK and GRGDNP, which have a higher specificity for the FnR (Pierschbacher and Ruoslahti, 1987); these peptides were also effective in inducing collagenase synthesis when immobilized.

We then tested whether larger fragments of Fn that contain the cell-binding domain and RGD sequence can mimic the inductive effects of the anti-FnR mAb. Collagenase and stromelysin expression was induced when RSF spread on immobilized 60- and 120-kD Fn fragments, in contrast to the lack of expression on immobilized native Fn (Fig. 8 *C*; Table I). The induction of metalloproteinases was dependent on the concentration of the immobilized Fn-derived peptides in the presence of collagen (data not shown). Although collagenase expression was also induced when the Fn fragments were added in solution (data not shown), we could not verify their monovalent status because of problems with aggregation of the fragments or their sticking to the surface of the culture dishes. Taken together, these data suggest that binding of Fn-derived peptides to the FnR triggers events different from those triggered by binding of native Fn.

Discussion

Our data demonstrate that perturbation of the interaction of Fn with its specific heterodimeric integrin receptor can alter gene expression. Treatment of attached and spread fibroblasts with an mAb against the specific integrin heterodimeric FnR, or with peptides containing the RGD cell-recognition sequence of Fn, but not with native Fn, induced expression of the genes for the metalloproteinases collagenase and stromelysin. Phorbol diesters, cytochalasins B and D, growth factors, and poorly adhesive substrates have also been shown to induce expression of these genes in fibroblasts (Aggeler et al., 1984*a*,*b*; Unemori and Werb, 1986, 1988; Edwards et al., 1987; Frisch and Ruley, 1987; Werb, 1989). The induction of proteinase expression by triggering the FnR and by these other treatments is similar in at least two respects. In all cases, there is a lag period before increased enzyme secretion is detectable. Once the inducing agent has been present for several hours, it can then be removed and the cells will go on to produce metalloproteinases over the next 24–48 h. Furthermore, proteinase induction by anti-FnR, as well as by the other inducers (Frisch and Ruley, 1987; Werb, 1989), is inhibitable by dexamethasone.

Despite these similarities, there is at least one important distinguishing feature between induction of proteinase expression mediated by the FnR and induction by previously reported treatments. Fibroblasts are induced to express metalloproteinases by anti-FnR even though they can remain flat and well spread throughout the induction period. Other treatments induce significant shape changes, which correlate with the extent of collagenase induction (Aggeler et al., 1984*b*; Werb et al., 1986). The divorcing of shape change from proteinase induction of collagenase and stromelysin gene expression by FnR has been documented in two ways. First, the organization of microfilament bundles, as detected by rhodamine-phalloidin, is not substantially altered during exposure to anti-FnR, whereas other agents, including the peptide containing RGD added in solution to cells spread in the presence of the adhesion proteins in serum, cause persistent and pronounced cell rounding and/or cytoskeletal reorganization. Although we cannot rule out the possibility that altered ligation of the FnR by the anti-FnR in solution induces more subtle or transient changes in cytoarchitecture, such as those seen in the first few minutes after the administration of growth factors such as epidermal growth factor



(Chinkers et al., 1979), in previous studies induction of proteinases was correlated with marked generalized alterations of the cytoskeleton of several hours' duration (Werb et al., 1986). In addition, studies on endothelial cells have shown that the FnR and Vn receptors are organized independently by their ligands, but both lead to local assembly of focal contacts and cytoskeletal proteins (Dejana et al., 1988). Thus, in cells exposed to serum, the cytoarchitecture may be maintained by the Vn receptor in the face of disruption of the FnR-Fn interaction. Second, proteinase expression by fibroblasts is stimulated even if they are cultured on substrates of anti-FnR, or of Fn-derived peptides containing RGD, that have been covalently linked to the culture dish. In this case, the fibroblasts spread on the mAb and the other immobilized inductive ligands as well as they do on noninductive collagen and Fn substrates and assume a highly flattened morphology, but are able to express proteinases. When Fn is conjugated to the dish, the cells spread but do not express proteinases. Thus, cells can distinguish whether they are attached to the substrate via their natural intact ligand or via Fn subfragments and the epitope recognized by the anti-FnR mAb, even though in all cases the cells have a similar morphology and degree of spreading. Because the selection screens for the anti-FnR mAb depended on inhibition of adhesion of cells to Fn, and peptides containing RGD also inhibit adhesion, it is likely that the anti-FnR mAb is a high affinity ligand for the subset of FnR configurations recognized by Fn subfragments. There are few data in the literature suggesting that Fn and Fn-derived peptides are recognized differently. However, monocytes, a cell type that expresses the FnR, show chemotaxis toward the cell-binding fragment of Fn but not native Fn in solution (Clark et al., 1988). Interestingly, these cells will respond to GRGDSP by activating their complement receptors only when bound to a surface (Wright and Meyer, 1985). Taken together, these observations suggest that the conformation of the binding site in Fn recognized by the FnR is likely to be altered when Fn is degraded by enzymes such as stromelysin (Chin et al., 1985).

There are other systems, such as adipocyte (Spiegelman and Ginty, 1983) and chondrocyte (Zanetti and Solursh, 1984) differentiation, in which the strong relationship between changes in cell shape and the induction of gene expression has also been observed. In these experiments, ECM molecules that reverse or prevent the shape change, such as Fn, prevent the induction of new gene expression. It is plausible that during induction of adipocyte-specific genes in preadipocytes and cartilage-specific genes in chondroblasts, as well as during induction of metalloproteinases in RSF by shape-altering reagents, the correlated reorganization of the actin cytoskeleton may act indirectly to alter adhesion of the cells via the FnR or other integrins. Therefore, these inductive signals may actually be transduced by the integrins.

Although RSF respond to anti-FnR whether it is presented in solution or substrate-bound, the two phenomena are fundamentally different in several respects and could be regulated by separate signaling mechanisms. In the first case, RSF are presented with soluble mAb after they have spread on the multiple adhesion proteins from serum and have formed a stable cytoskeletal framework. The effect of the soluble mAb would then be expected to be directly inductive for gene expression. In contrast, when freshly trypsinized, rounded RSF, with a disorganized cytoskeleton and diffuse FnR distribution, are plated on the immobilized anti-FnR, it is the interaction with mAb, not the native adhesion ligands, that induces the spreading of the cells. Because cell rounding and actin disorganization are correlated with metalloproteinase expression, it is possible that, upon exposure of cells to the native ligand, the inhibitory signal usually generated by interaction of Fn and other adhesion proteins with integrins stops the induction of metalloproteinases. However, when RSF are spreading on anti-FnR or Fn-derived peptides, the inhibitory signal does not go into effect, and gene expression is induced by a default pathway. These mechanisms remain to be explored.

Our data showing that anti-FnR and peptides containing RGD are inductive for metalloproteinase gene expression when multimeric or immobilized, but not when in solution in monovalent form, suggest that aggregation of the receptor is a prerequisite for transfer of information. The effects are specific to the FnR; cross-linking of other glycoprotein receptors such as the LDL-R and transferrin receptor did not induce the metalloproteinases. Although the distribution of the FnR on RSF was not determined directly in the present study, because this anti-FnR mAb does not stain well, there is a correlation between receptor aggregation and receptor function for two other integrins, Mac-1 of the β_2-subclass and GPIIb/IIIa of the β_3-subclass (Detmers et al., 1987; Isenberg et al., 1987). Therefore, cross-linking of the FnR may constitute part of the mechanism for transducing the signal for collagenase induction as it does for a variety of other receptors, including those for insulin (Kahn et al., 1978) and epidermal growth factor (Wakshull and Wharton, 1985). It is of interest in this regard that a fibrinogen decapeptide is able to induce aggregation of GPIIb/IIIa in platelets. However, aggregation alone clearly does not trigger the biological response, because the FnR is aggregated in focal contacts

Figure 8. Induction of metalloproteinase expression by peptides containing an RGD sequence. (*A* and *B*) Monolayers of RSF cultured to confluence in medium containing serum in 48-well plates were left untreated (lane *1*) or incubated with various concentrations of the GRGDSP peptide (lanes *2–4*), which contains the cell adhesion recognition sequence for integrins, or with the control GRGESP peptide (lanes *5–7*) in DME-LH for 24 h. The cell shape indices of the treated RSF in lanes *1–7* were 0, 1, 2, 3, 0, 0, and 0, respectively. (*A*) Samples (10 μl) of the CM were then analyzed for secreted proteinases by zymography on an SDS-gelatin substrate gel. (*B*) The cells were then biosynthetically labeled with [^{35}S]methionine for 4 h and the secreted proteins were analyzed by SDS-PAGE followed by fluorography. (*C*) RSF were plated on coverslips coated with immobilized proteins or peptides prepared by covalently cross-linking type I collagen at 1 mg/ml (lane *1*), collagen at 1 mg/ml plus GRGESP at 40 μg/ml (lane *2*), collagen at 1 mg/ml plus GRGDSP at 40 μg/ml (lane *3*), GdRGDSPASSK at 200 μg/ml (lane *5*), native Fn at 10 μg/ml (lane *6*), 120-kD fragment of Fn at 10 μg/ml (lane *7*), 60-kD fragment of Fn at 10 μg/ml (lane *8*), or anti-FnR mAb at 140 μg/ml (lane *9*). Lane *4*, RSF plated on plain glass coverslips. Some of the samples are shown in duplicate. After incubation for 30 h (lanes *1–3*) or 34 h (lanes *4–9*) in DME-LH, the cells were biosynthetically labeled with [^{35}S]methionine for 4 h and the secreted proteins were analyzed by SDS-PAGE followed by fluorography. Molecular weight standards ($\times 10^{-3}$) and the migration of procollagenase (*proCL*) and prostromelysin (*proSL*) are indicated.

when cells adhere to Fn, but no collagenase is induced under those conditions. Thus, it is likely that the inductive ligands produce a change in receptor conformation and/or interaction with the membrane along with oligomerization of the receptor and that this change contributes to signal transduction. The anti-FnR mAb and Fn-derived peptides induce a different subset of signals and responses than does the natural ligand Fn. In platelets, it is possible that different ligands induce different conformations of GPIIb/IIIa (Phillips et al., 1988). Taken together, all these results point to the conclusion that the nature of the interaction of the FnR with its ligand is an important factor in regulating gene expression. Thus, in addition to mediating cell attachment and spreading on Fn and formation of a membrane–actin cytoskeleton complex, the FnR complex can mediate signal transduction between the external environment and the cell interior. The nature of the signaling molecules is currently under investigation.

The integrins are good candidates to be involved in signal transduction. Integrins are transmembrane heterodimers that interact directly with their ECM ligands (Horwitz et al., 1985; Pytela et al., 1986; Tomaselli et al., 1988; Gehlsen et al., 1988*b*; Gailit and Ruoslahti, 1988) and with talin, a molecule associated with the cytoskeleton (Burridge, 1986; Buck et al., 1986). Tyrosine phosphorylation on the β_1-chain may regulate affinity for both Fn and talin (Burridge, 1986; Hirst et al., 1986; Buck and Horwitz, 1987). Our data suggest that integrins can act as a kind of homeostatic system for modulating ECM structure and organization in response to the needs of the cell. The high affinity interactions of mAbs with integrins mimic the interactions of integrins with fragments of ECM ligands, and thus make them useful for studying the pleiotropic ligands and functions of these receptors.

ECM remodeling is particularly important during embryonic development, in wound healing, in chronic inflammation, and in metastasis and embryo implantation (Fairbairn et al., 1985; Mignatti et al., 1986; Schultz et al., 1988; Sutherland et al., 1988). As a result of wounding, for example, there are likely to be significant changes in the environment of the cells at the wound site, including generation of fragments of ECM ligands, with an ordered series of events requiring ECM degradation, cell migration, ECM resynthesis, and remodeling. The pleiotropic responses of cells to interaction of their integrins with a changing population of ligands may therefore not only reflect the changes in the cellular environment but may actively mediate them through changes in expression of ECM and EMC-degrading molecules.

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BBA Report

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Porous glass as a solid support for immobilisation or affinity chromatography of enzymes

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SUMMARY

Chymotrypsin (EC 3.4.4.5) and β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) were immobilised by attachment with glutaraldehyde to aminoalkylsilyl glass. The preparations contained 16 and 12 mg protein/g glass respectively. The retention of activity on immobilisation for chymotrypsin was 50% and for β-galactosidase was 36%. Glycyl-D-phenylalanine was immobilised in the same way and used for purification of carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.1.2) by affinity chromatography.

Support materials such as cellulose, polyacrylamide, and dextran derivatives used for enzyme immobilisation and affinity chromatography are not ideal for large-scale operation in columns. The attachment of biologically active molecules to porous glass beads[1] is therefore of interest. They are resistant to microbial attack and can be regenerated by heating to remove all organic material. Here we describe a cheap and rapid method of attachment of enzymes and enzyme inhibitors to porous glass beads using glutaraldehyde.

Porous glass beads (Corning CPG 10, 200 mesh, 2000A pore diameter) were dried by heating at 500°C for 6 h. The method used by Weetall[1] for the preparation of amino-alkylsilane glass was optimised to give higher yields of amino groups/g of support. These were measured with 0.1% trinitrobenzene sulphonic acid in saturated sodium tetraborate[2], and found to be 30–35 μmoles NH_2/g support. The glass was refluxed in 0.1% 3-amino-propyltriethoxysilane in toluene for 36 h. Another method however gave better results. The beads were immersed in a 2% solution of 3-aminopropyltriethoxysilane in acetone. Excess liquid was decanted off and the beads allowed to stand at 45°C for 24 h. This direct polymerisation produced beads containing 80–90 μmoles NH_2/g support, and was used in preparing the derivatives described in this paper.

Aminoalkylsilane glass (2 g) was stirred in a cold 1% aqueous solution of

glutaraldehyde for 30 min. The derivative was rinsed with water and suspended in 10 ml of 0.05 M phosphate buffer, pH 7.5, containing 40 mg of α-chymotrypsin (EC 3.4.4.5) (Miles–Seravac (PTY) Ltd., U.K.). After 2 h at 4°C the beads were washed thoroughly with 1 M NaCl until no further activity was detectable in the washings. The immobilised chymotrypsin was assayed using *N*-acetyl-L-tyrosine ethyl ester as described by Kay and Lilly[3]. The immobilised preparation contained 16 mg of chymotrypsin/g support. When assayed in the absence of buffer the immobilised chymotrypsin had an optimum at pH 9.5 of 2.0 μmoles/min per mg support, corresponding to a retention of enzyme activity of about 50%. Addition of 0.01 M phosphate buffer to the assay mixture shifted the pH–activity profile giving a broad optimum between pH 8 and 9, similar to that for the free enzyme. This effect was similar to that previously observed with chymotrypsin attached to DEAE-cellulose[3]. The glass–chymotrypsin derivative was more heat stable than the free enzyme. When incubated at pH 3 for 2 h at 50°C the derivative lost 8% of its activity. In an identical experiment at pH 8, 19% of the activity was lost.

β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) was attached to glass beads by a similar procedure to that used for chymotrypsin. An ammonium sulphate fraction from an extract of *Escherichia coli* was dialysed against 0.01 M Tris buffer containing 0.01 M $MgCl_2$ and 0.01 M 2-mercaptoethanol at pH 7.5. Aminoalkylsilylglass beads were activated with glutaraldehyde, washed and then reacted with β-galactosidase solution for 2 h at 4°C. The product was washed with the above buffer solution which is also suitable for storing the immobilised enzyme. The glass–β-galactosidase contained 12 mg protein/g glass. When assayed with *o*-nitrophenyl galactoside at 25°C and pH 7.5 the preparation had an activity of 0.13 μmoles/min per mg support, corresponding to a retention of activity of 36%. The immobilised β-galactosidase lost no activity at room temperature for 3 days when 0.01 M mercaptoethanol was present.

An inhibitor of carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.2.1), the dipeptide, glycyl-D-phenylalanine, was coupled to porous glass by suspending 2 g of the glutaraldehyde-activated glass in 5 ml of phosphate buffer, pH 7.5, containing 25 mg of the dipeptide. The suspension was stirred for 24 h at room temperature and subsequently washed with 2 M NaCl. After being packed in a column the glass derivative was equilibrated at 25°C with 0.02 M Tris buffer, pH 7.5, containing 0.2 M NaCl. The column was charged with 1 ml of a solution containing 2 mg of carboxypeptidase A (Sigma London Chemical Co.) and 8 mg of a mixture of lysozyme, ribonuclease and bovine serum albumin. The column was washed through with the starting buffer. The bound carboxypeptidase A was eluted from the column with 0.01 M acetic acid, pH 3.0. Carboxypeptidase A activity in the column effluent was determined by the colorimetric procedure of Ravin and Seligman[4].

The results for a small column (0.5 cm × 4 cm) are shown in Fig. 1. The flow rate was 7.5 ml/h. The recovery of protein was 96% and of carboxypeptidase A activity was 80%, 10% of the protein and 85% of the recovered activity was in the fraction retained by the column. The best preparations of dipeptide glass removed 2 mg of enzyme from solution for each g of glass. α-Chymotrypsin, which is inhibited by D-amino acids[6], exhibited some affinity for the column.

The attachment of enzymes to porous glass by diazotisation of an arylamine derivative and by sulphonamide linkage using an isothiocyanate derivative has been reported by Weetall[1]. Glutaraldehyde has been used to cross-link enzymes to form insoluble

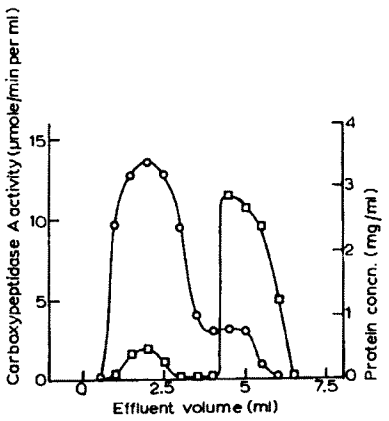

Fig. 1. The separation of carboxypeptidase A from a mixture of proteins by affinity chromatography. ○—○, protein; □—□, carboxypeptidase activity. 0.01 M acetic acid was added after 4 ml of effluent had been collected.

aggregates[5] and to immobilise enzymes to solid supports such as AE-cellulose[6]. Here we have shown that it may be used to link enzymes and an enzyme inhibitor to porous glass in three simple steps. It should be pointed out that glutaraldehyde solutions normally contain condensation products[7], and it is not known which of these is the active species. The two enzyme preparations retained a high proportion of their activity on immobilisation. The amount of protein bound to the glass was higher than in most of the preparations previously described[1]. However, as might be expected with the highly active chymotrypsin derivative, there was some sign of diffusional limitation of the reaction rate including a shift in the pH optimum. Diffusional limitation may account for part of the decrease in activity on immobilisation.

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